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THE ABSORPTION OF VOLATILE FATTY ACIDS FROM THE RUMEN

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(With Three Text-figures)

The fermentation of carbohydrates in the rumen of sheep is known to lead to the formation of large amounts of the lower fatty acids. The important part that these acids assume in the metabolism of ruminants has been remarked upon (Marston, 1939, 1946). While it is believed that acetic, propionic and butyric acids are the main fatty acid products of the fermentation, there is no certain knowledge of the proportions and quantities in which they are formed.

There is evidence that absorption may proceed directly from the rumen (Trautmann, 1933; Davey, 1936; Phillipson & McAnally, 1942), and more recent work (Barcroft, McAnally & Phillipson, 1944) has demonstrated that fatty acids can find their way through the rumen wall directly into the blood stream. This being so, a knowledge of the relative proportions of the fatty acids in the rumen contents may, of itself, provide no indication of the proportions in which they are formed, as it would not be improbable that the individual fatty acids would be absorbed at different rates.

It is clear, therefore, that a determination of the relative rates of absorption of the acids is required for a better understanding of the proportions in which the acids are formed in the rumen, and absorbed from it. Barcroft *et al.* (1944) presented evidence which was interpreted as elucidating this point. The case depended essentially on an increase of volatile fatty acids observed in the efferent blood from part of the rumen wall of an anaesthetized animal, after one or other of the acids had been introduced into the empty rumen. The composition of the lymph draining this organ was not determined, and for this reason alone the interpretation of the findings might be complicated; the possibility of differential absorption into the blood and lymph respectively is by no means remote.

In order to check this point the feasibility of collecting from the rumen lymphatics sufficient material for critical chemical analysis was considered. Owing to the technical difficulties involved, however, this means of approach was abandoned in favour of the direct determination of relative rates of disappearance of the lower fatty acid homologues when introduced into the empty, washed rumen as mixtures in dilute aqueous solution approximating the composition of the contents of this organ when active digestion is in progress. It was thought that this might provide less equivocal evidence.

The soundness of such a procedure would depend essentially on two factors; the precise estimation of individual fatty acids in mixtures of this sort, and the proof that residual bacterial activity would be reduced to such a low level as to prevent appreciable decomposition of any of the components of the mixtures employed. The former point has been dealt with in another publication (Gray, 1947); evidence to dispel the latter possibility as a complication of the experiments described here is presented with the other data.

EXPERIMENTAL

In the following trials a 6-year-old sheep, in which a permanent rumen fistula had been established nearly 2 years previously, was used. The rumen was emptied through the fistula, washed out with a large volume of warm water, and the residual water removed as completely as possible by siphoning. The test solution was then introduced into the rumen and samples were drawn off for analysis at intervals. The size of the samples varied according to the amount of fatty acid present.

The volatile fatty acids were first separated from other substances by distillation with steam according to the procedure of Osburn, Wood & Werkman (1936). The methods employed for the determination of the individual fatty acids in the distillate have been discussed elsewhere (Gray, 1947). Individual acids, when only two were present, were determined by the semi-distillation method of Virtanen & Pulkki (1928). When acetic, propionic and butyric acids were all present, the butyric acid was determined by the method of Klinc (1934) and the other two acids by semi-distillation, allowance for the butyric acid being made in the equations.

Trials nos. 1 *and* 2

In each of the first two trials a mixture of acetic, propionic and butyric acids was neutralized to phenolphthalein with NaOH and between 3 and 4 l. of the resulting solutions were introduced into the empty rumen as described. Results of the analyses of samples drawn over 6 hr. periods are given in Table 1.

Table 1. *Composition of mixtures of fatty acids introduced into the empty rumen*

Time after introduction hr.	Total acid g./100 ml.	% acetic	% propionic	% butyric
Trial 1				
—	1·20	34·5	42·3	23·2
¼	0·882	36·1	41·5	22·4
¾	0·729	34·6	43·1	22·3
1¾	0·671	36·2	40·3	23·5
3¼	0·515	35·5	43·1	21·4
4¾	0·420	30·0	45·0	25·0
6¼	0·391	33·0	44·5	22·5
Trial 2				
—	1·50	49·9	36·1	14·0
¼	1·38	50·2	35·4	14·4
1	1·13	52·2	35·7	12·1
Animal fed in this period				
3	0·787	75·9	14·6	9·5
5	0·583	93·1	—	6·9
6	0·526	95·8	—	4·2

In trial 1 the total acid concentration fell to about one-third of its original value in 6 hr., but the relative proportions of the acids present did not appear to change significantly in that time. In trial 2 a somewhat stronger solution was introduced, and the total acid concentration again fell to about one-third of its initial value in 6 hr. During this experiment, however, the animal contrived to escape and eat some lucerne. Samples drawn after it had been returned to its pen all indicated a marked progressive change in the relative proportions of the acids present. Propionic acid disappeared within 4 hr. and the proportion of butyric acid fell, leaving a large preponderance of acetic acid. The data clearly indicated a more rapid removal of propionic acid under these conditions.

When the conditions of this experiment were repeated in further trials it was again found that, after feeding, the relative concentrations of the acids changed, and though not so pronounced as in trial 2, these changes indicated a more rapid loss of propionic acid.

In view of the fact that the rumen had been thoroughly washed out just before the trials started the eating of lucerne is unlikely to have been followed by any appreciable fermentation within the short time of the experiment. It was possible, however, that it had resulted in the addition of a considerable amount of saliva to the rumen contents, and that this might be a factor in the changed behaviour towards the acids. An exploratory trial showed that the inclusion in the test solution of an amount of inorganic phosphorus (as NaH_2PO_4) approximately equivalent to that found in sheep's saliva (Watson, 1933), led to changes in composition similar to those occurring when the animal had been allowed to feed during the experiment.

It was not thought that the presence of phosphate was essential for the absorption of fatty acids by the rumen; in fact, it has been demonstrated that absorption takes place from solutions of sodium acetate and propionate alone (Barcroft *et al.* 1944), but because the solutions used in this latter case contained the salts in materially greater concentrations than were present in trial 1, it was considered likely that differential absorption from more dilute solutions was too slow to be appreciable. In the presence of phosphate, however, equally dilute solutions underwent changes in composition indicating absorption, at least of propionic acid. It appeared possible that the presence of phosphate, which is a constituent of the normal rumen contents, had increased the rate of absorption of the acids, and so phosphate was added to the test solutions in the next series of trials, without at this stage making further investigation of its function.*

Determination of the relative rates of absorption of acetic and propionic acids from the rumen. While there was a very considerable decrease in total acid concentration in trial 1, some or all of this fall may have been due to dilution. In trial 2 loss of acid by absorption was clearly indicated by the changed proportions of the acids present;

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* Experiments conducted since this was written have shown that propionic acid is absorbed preferentially from very dilute solutions containing no phosphate and adjusted to *p*H 6·5 before they were introduced into the rumen. Little if any preferential absorption of propionic acid was observed when more concentrated solutions were introduced at the *p*H used in trial 1 even if phosphate were present. Further work is proceeding to determine the influence of *p*H on the absorption of fatty acids from the rumen.

but because the rumen contents may also have been diluted an estimate of the relative rates of absorption could not be made. In order to do this an inert 'marker' which is not absorbed was employed as a reference material for the assessment of the degree of dilution. Pectin was chosen since it was most unlikely to be absorbed, it could be dispersed completely in the test solutions, and its concentration could be determined with relative ease.

As it was necessary to decide whether there was sufficient residual bacterial activity in the washed rumen to decompose any of the components of the solutions used, a test was made. Four litres of a solution containing sodium acetate, sodium propionate and pectin, in which the final reaction had been adjusted to *p*H 6·5, were introduced into the washed rumen. After 1 hr., when it was considered that thorough mixing of the rumen contents had taken place, a large sample was withdrawn. One portion was used immediately for analysis; the remainder was incubated for 5 hr. at 38° C. in a completely filled, closed flask, before analysis was made. The test was then repeated with a solution containing 12 g. NaH_2PO_4 in addition to the previous components. The analytical results shown in Table 2 indicate that there was not sufficient residual bacterial activity in the washed rumen to cause significant decomposition of any of the components of the mixtures within 5 hr.

Table 2

Components	Solution (1) no phosphate		Solution (2) phosphate present	
	Before incubation g./100 ml.	After incubation g./100 ml.	Before incubation g./100 ml.	After incubation g./100 ml.
Acetic acid	0·530	0·556	0·627	0·612
Propionic acid	0·493	0·479	0·474	0·492
Pectin (as Ca pectate)	0·250	0·246	0·349	0·345

Trials nos. 3–5

In each of the following trials, 4 l. of a test solution were introduced into the emptied, washed rumen. Each mixture contained two acids, acetic and propionic, together with about 6·5 g. NaH_2PO_4 and 15 g. pectin. A small amount of $NaHCO_3$ added during the neutralization ensured the presence of CO_2 in trials 3 and 4, but it was omitted in trial 5. The final reaction was adjusted to about *p*H 6·5.

In the original solutions and in the samples drawn, pectin was determined by the calcium pectate method of Nanji & Norman (1928). Data from the three trials are summarized in Table 3.

From column (3) in this table it is clear that, as in the earlier trials, propionic acid was absorbed more rapidly than acetic acid. The proportions of the acids present in the rumen contents in each trial have been plotted against the sampling times in Fig. 1.

From the concentrations of pectin (expressed as Ca pectate) in column (6) it is evident that a considerable dilution of the rumen contents took place in each trial. The relative dilution of the rumen contents during the trials is shown in Fig. 2,

Table 3. *The composition of mixtures of fatty acids and pectin introduced into the empty rumen*

[An extra figure has been retained throughout the data to minimize accumulation of arithmetical errors.]

Trial 3

Time after introduction	Total acid g./100 ml.	Acetic %	Propionic %	Acetic g./100 ml.	Propionic g./100 ml.	Ca pectate g./100 ml.	g./0·350 g. Ca pectate	
							Acetic	Propionic
(1)	(2)	(3)		(4)	(5)	(6)	(7)	(8)
—	1·52	39·7	60·3	0·603	0·922	0·350	0·603	0·922
10 min.	1·30	40·0	60·0	0·520	0·780	0·298	0·610	0·916
1 hr.	1·04	41·1	58·9	0·429	0·616	0·281	0·534	0·766
2 hr.	0·870	45·4	54·6	0·395	0·475	0·276	0·501	0·602
4 hr.	0·665	51·1	48·9	0·340	0·325	0·247	0·482	0·460
6 hr.	0·505	56·4	43·6	0·285	0·220	0·219	0·455	0·351

Trial 4

Time after introduction	Total acid g./100 ml.	Acetic %	Propionic %	Acetic g./100 ml.	Propionic g./100 ml.	Ca pectate g./100 ml.	g./0·397 g. Ca pectate	
							Acetic	Propionic
(1)	(2)	(3)		(4)	(5)	(6)	(7)	(8)
—	1·78	37·2	62·8	0·663	1·12	0·397	0·663	1·12
10 min.	1·63	36·8	63·2	0·600	1·03	0·377	0·632	1·09
1 hr.	1·18	38·0	62·0	0·450	0·734	0·307	0·582	0·949
2 hr.	0·929	41·2	58·8	0·383	0·546	0·274	0·554	0·791
4 hr.	0·678	48·6	51·4	0·329	0·349	0·247	0·529	0·561
6 hr.	0·600	51·0	49·0	0·306	0·294	0·238	0·510	0·490

Trial 5

Time after introduction	Total acid g./100 ml.	Acetic %	Propionic %	Acetic g./100 ml.	Propionic g./100 ml.	Ca pectate g./100 ml.	g./0·408 g. Ca pectate	
							Acetic	Propionic
(1)	(2)	(3)		(4)	(5)	(6)	(7)	(8)
—	0·894	59·3	40·7	0·530	0·364	0·408	0·530	0·364
1 hr.	0·552	63·4	36·6	0·350	0·202	0·353	0·404	0·231
2 hr.	0·387	69·0	31·0	0·267	0·120	0·320	0·340	0·153
3 hr.	0·258	77·5	22·5	0·200	0·058	0·252	0·324	0·094

where the concentration of pectin at the start of each trial has been taken as 100, and the subsequent concentrations calculated in proportion. The rate of addition of saliva appears not to be constant except in trial 5, but at the start of trials 3 and 4 there may have been larger amounts of water left in the rumen after washing, and this could account for the more rapid dilution at that time.

It is evident that the absolute rates of absorption of the acids may not be assessed without a knowledge of the amounts of pectin and acids which have passed on into the abomasum during the various stages of the experiments. But provided pectin is not absorbed through the rumen wall, then the relative rates of absorption of the acids can be estimated by comparing changes in composition in relation to a constant amount of the inert marker, pectin. For this purpose, changes in the concentrations of the acids in terms of the original concentrations of pectin are shown in the last two columns of Table 3. In Fig. 3 the concentrations from columns 4, 5, 7 and 8 of trial 3 in the table have been plotted against the sampling times. From the curves the losses of each acid relative to the constant amount of pectin may be calculated over a particular range of acid concentration in the rumen. For example, from Fig. 3,

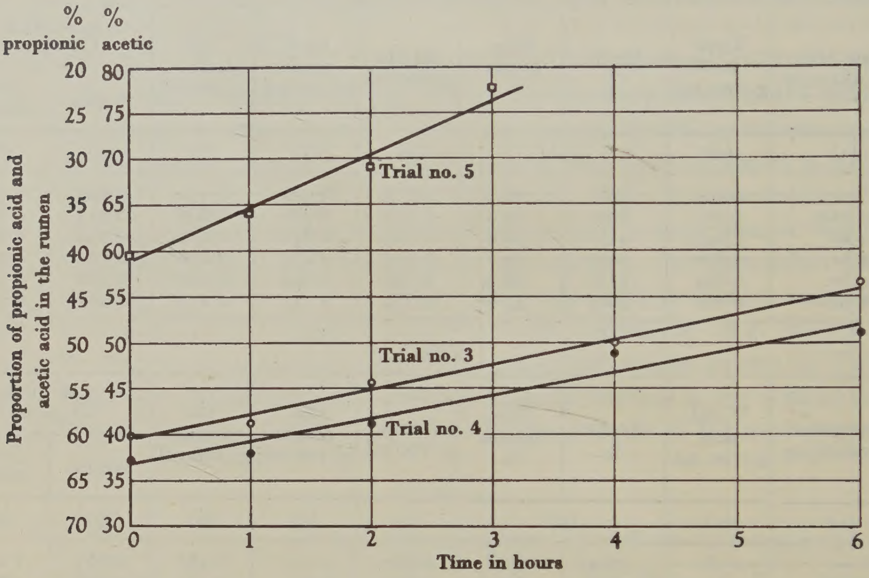

Fig. 1. Proportions of acetic and propionic acids in the rumen contents during trials 3, 4 and 5.

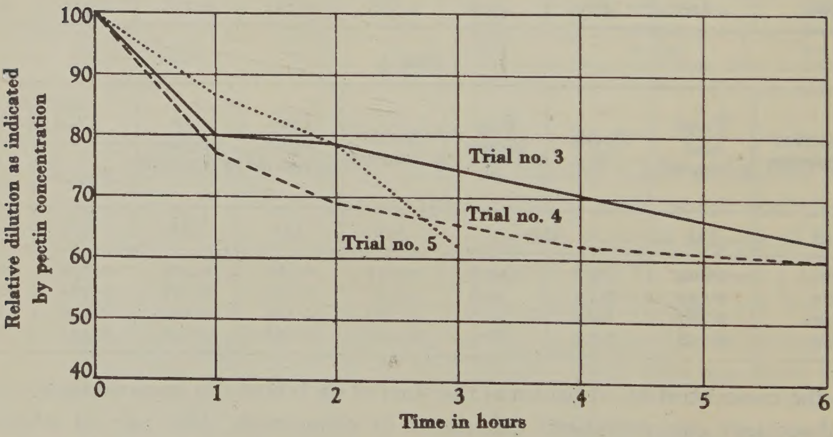

Fig. 2. Relative dilution of rumen contents during trials 3, 4 and 5. The pectin concentration at the start of each trial has been made 100.

over the range of concentration between 0·3 and 0·6 g. acid/100 ml., the loss of acetic acid was 0·150 g./unit of marker (0·35 g. Ca pectate) in 5·75 hr., which signifies an average absorption rate of 0·026 g. acetic acid/hr., and the loss of propionic acid was 0·325 g./unit of marker (0·35 g. Ca pectate) in 3·50 hr., which signifies an average absorption rate of 0·093 g. propionic acid/hr. The ratio of the rates of absorption of propionic acid: acetic acid then is 3·6.

The ratios calculated in this way from all three trials for a series of concentration ranges are shown in Table 4.

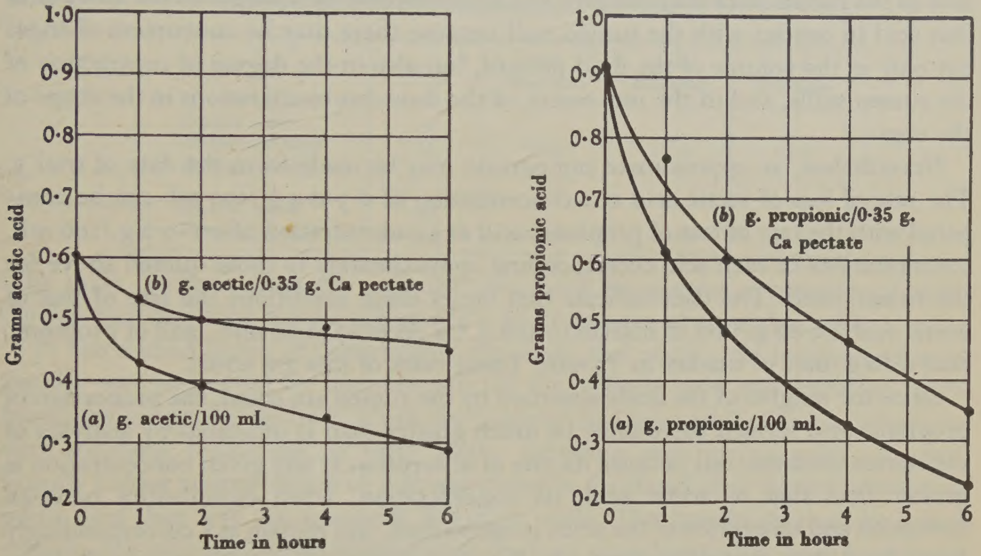

Fig. 3. Concentrations of acetic and propionic acids in the rumen contents during trial 3. The concentrations of each acid are expressed first in g./100 ml. of rumen contents in curve (*a*), and secondly in g./0·35 g. of Ca pectate in curve (*b*). For any given range of acid concentration in the rumen the losses of acetic acid and propionic acid in relation to a constant amount of pectin (0·35 g. Ca pectate) can be read off, together with the period of time in which they took place. In this way the relative rates of absorption of the two acids at the given range of concentration are determined.

Table 4

Range of concentration g. acid/100 ml.	Ratio $\frac{\text{Rate of absorption of propionic acid}}{\text{Rate of absorption of acetic acid}}$		
	Trial 3	Trial 4	Trial 5
0·2–0·3	—	—	4·0
0·3–0·4	4·9	4·8	—
0·4–0·5	2·1	3·0	—
0·5–0·6	1·6	1·7	—

Over a range of concentration of 0·2–0·6 g./100 ml., propionic acid was absorbed more rapidly than acetic acid, and at the lower concentrations the relative rate of the absorption of propionic acid was much greater than at the higher concentrations.

In view of this variation with concentration it may be stressed here that a large number of analyses of the rumen fluid of a sheep fed on wheaten-hay chaff, indicated a range of concentrations of total volatile fatty acid from 0·37 to 0·64 g./100 ml., and that, of this acid, acetic acid constituted approximately 60%, while the proportion of propionic acid varied from 10 to 30%.

From the data available from these trials no exact comparison can be made between the rate of absorption of acetic acid at one concentration and that of propionic acid at another. To do this the relationship between the concentration of each acid and its rate of absorption would need to be accurately established. This relationship may be complicated by the fact that changes in the concentration of an acid in the rumen fluid may not give rise to corresponding changes in the amount of that acid in contact with the rumen wall because there may be concurrent changes not only in the volume of the fluid present, but also in the degree of contraction of the rumen walls, and in the movement of the fluid due to alterations in the shape of the organ.

Nevertheless, an approximate comparison may be made from the data of trial 5. The rate of loss of acetic acid at a concentration of 0·3–0·4 g./100 ml. can be compared with the rate of loss of propionic acid at a concentration of 0·1–0·2 g./100 ml., concentrations of each acid corresponding approximately to those quoted above for the rumen itself. The data indicate that under these conditions the rate of loss of acetic acid is 0·08 g./unit of marker (0·408 g. Ca pectate) in 60 min., and of propionic acid 0·10 g./unit of marker in 75 min. These rates of loss are equal.

Since the weights of the acids absorbed by the rumen are equal, the proportion of propionic acid formed there must be much greater than is indicated by analyses of the rumen contents, but because its rate of absorption at any given concentration is greater than that of acetic acid, its concentration, when equilibrium between formation and absorption of the acids is established, will remain at a correspondingly lower level than that of acetic acid. Propionic acid is formed in relatively large proportions in the rumen during the fermentation of wheaten-hay chaff; it is found present in much smaller proportions only because it is more readily absorbed than acetic acid.

DISCUSSION

From the above data it may be concluded that propionic acid is absorbed preferentially when a mixture of acetic and propionic acids is introduced into the rumen. The findings are thus in conflict with the conclusions of Barcroft *et al.* (1944) which suggest that acetic acid is absorbed from the rumen more rapidly than propionic acid. This latter claim was based on two series of experiments. In the first of these, comparisons were made of the volatile acid concentrations in samples taken at approximately the same time from the contents of the rumen and from the blood draining its walls. The solutions of mixed acids were not subjected to exact analysis, but were distilled under similar conditions and the rates of distillation compared. Because on redistilling the distillate from the contents of the rumen the early fractions contained more acid than the corresponding fractions from the blood draining its walls, it was assumed that the proportion of acetic acid to higher acids in the former was less than in the latter, and so it was concluded that acetic acid was being absorbed more rapidly than the higher fatty acids. This assumption is not valid, even if the conclusion drawn from it is correct. The presence of butyric acid in the rumen contents would render questionable conclusions based on this method of approach. It is not difficult to conceive mixtures of fatty acids in proportions that might well occur in the blood and rumen respectively, which, if treated and the results interpreted in this way, would lead to an erroneous conclusion. For example, a distillate from rumen contents containing a mixture of fatty acids in the molecular proportions of acetic acid 50%, propionic acid 20%, and butyric acid 30%, and one from blood containing fatty acids in the proportion of acetic acid 45%, and propionic acid 55%, when distilled in this fashion would yield the following data:

	%	1st fraction	2nd fraction	3rd fraction
Acetic acid Propionic acid Butyric acid	50 20 30	20·3	17·4	14·7
Acetic acid Propionic acid	45 55	15·8	15·8	14·9

The early fractions from the mixture containing the greater proportion of acetic acid in this case then have a *higher* not a *lower* amount of titratable acid.

In the second series of experiments the claim that acetic acid is absorbed more rapidly was supported by measurement of the volatile acid concentration in the blood draining a rumen from which the contents had been washed out and replaced with solutions containing, in turn, acetic, propionic and butyric acids. A significantly higher concentration of acid was found in the blood after the introduction of acetic acid than was the case with the other acids. The possibility that the lymph vessels are a normal channel of absorption for fatty acids has already been mentioned. A more rapid absorption of acetic acid into the blood stream, then, need not necessarily imply a more rapid passage of acetic acid through the rumen wall. The concentrations of sodium acetate and sodium propionate employed by Barcroft *et al.* were greater than those used in the experiments reported in this paper, and as it has been shown that the rate of absorption of propionic acid relative to that of acetic acid decreases with increasing concentrations of the acids, there may not be any conflict between the present findings and those subjected to the critical discussion above. The more direct approach, however, has shown unequivocally that propionic acid is absorbed preferentially through the rumen wall when mixtures containing acetic and propionic acids in concentrations usually found in the rumen contents are introduced into the emptied, washed rumen.

SUMMARY

1. When a dilute solution of the sodium salts of acetic, propionic and butyric acids was introduced into an empty rumen no significant change was observed in the relative proportions of the acids present, within a 6 hr. period.

2. When a similar mixture was introduced, and the animal ate lucerne during the experiment, marked changes occurred in the proportion of the acids present, indicating a more rapid absorption of propionic acid than of the other acids.

3. A similar absorption was observed on introducing a solution of the sodium salts of acetic and propionic acid, at *p*H 6·5, containing an amount of inorganic phosphorus equivalent to that occurring in sheep's saliva. By the inclusion of pectin as an unabsorbed reference substance in such a mixture it was shown that a considerable dilution of the rumen contents occurred during the experiment—presumably by the advent of saliva. The relative rates of absorption of acetic and propionic acids were determined over particular ranges of acid concentrations.

4. On the basis of the relative rates of absorption of these acids at concentrations in which they were found to occur in the normal rumen during the fermentation of wheaten-hay chaff, it was concluded that a much greater proportion of propionic acid is formed than is indicated by analyses of the acids found in the rumen.

The author is indebted to Mr H. R. Marston, Chief of the Division, for his help and encouraging interest throughout the course of these investigations. His thanks are due also to Miss B. du Rieu for her assistance, and to Mr I. G. Jarrett for the establishment of the fistula in the experimental animal used.

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Since this work was prepared for publication, a paper has come to hand by Danielli, Hitchcock, Marshall & Phillipson (1945), in which they have described the mechanism of absorption of volatile fatty acids from the rumen. The present paper covers a part of the same field and provides confirmation of some of their findings, by different methods.

DANIELLI, J. F., HITCHCOCK, M. W. S., MARSHALL, R. A. & PHILLIPSON, A. T. (1945). *J. Exp. Biol.* **22**, 75.

THE ANALYSIS OF MIXTURES OF VOLATILE FATTY ACIDS

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In the course of investigating certain aspects of carbohydrate fermentation in the rumen of the sheep it became necessary to estimate the individual concentrations of formic, acetic, propionic and butyric acids in aqueous solutions containing all four components.

Two main procedures are available for such analyses: the distillation method of Hillig & Knudsen (1942), which is based essentially on the classical Ducleaux (1874, 1900*a*, *b*, 1908) procedure, and the method of Osburn, Wood & Werkman (1936) which involves partition of the acids between water and ether phases. In each of these methods formic acid is estimated by a separate procedure.

Preliminary examination of the partition method proved its effectiveness for the assay of mixtures containing acetic, propionic and butyric acids except when one of the components constituted either a very large or a very small proportion of the mixture. As it was expected that such mixtures would be encountered, an improvement in the method was sought.

No difficulties were experienced in the analysis of solutions prepared from mixtures of two acids, when one of them was present in much larger proportion than the other; but when analyses were made of mixtures containing known concentrations of three acids, the amounts of each, determined by the partition procedure, indicated that serious sources of error are inherent in the method. This is to be expected; the estimation involves essentially a calculation by difference and so in certain circumstances the implied error is large. Assuming, for example, an accuracy of ±0·5% in the titration of the acid content of the aqueous phase of each partition, and of ±0·1% in the titration of the original acid mixture, the theoretical maximum error involved in the estimation of propionic acid in a mixture containing acetic acid 1·5 g./l., propionic acid 0·25 g./l. and butyric acid 0·25 g./l. would be 67%; whereas, with the same manipulative accuracy, the maximum error involved in the estimation of this component of a binary mixture containing only acetic and propionic acids in the above concentrations would be reduced to 10%.

If the concentration of butyric acid could be determined by a separate estimation with an accuracy of even 5%, which may readily be accomplished, then, by applying the partition procedure and substituting the figure for butyric acid in solving the equations, the maximum error involved in the estimation of the propionic acid in the former mixture would be reduced to 19%.

It is clear, therefore, that the accuracy of the determination of the concentrations of each of three acids in a mixture should be very considerably increased if the concentration of one of them is determined by an independent method and the resulting data introduced prior to solving the partition equations. It is also clear that if such a combined procedure is to improve the precision of the method, the accuracy of the independent determination of the third acid must increase with the accuracy of the measurement of the partitions.

The case for improved accuracy arising from the use of combined methods of this sort would apply equally well to both partition and distillation procedures, and so the method of distillation at constant volume (Hillig & Knudsen, 1942), or, since the collection of only one fraction of distillate would be necessary, the simpler semi-distillation procedure (Virtanen & Pulkki, 1928) could be adopted. In spite of the fact that the distillation constants of the acids are less widely different than are their partition constants, such a combination of a distillation method for acetic and propionic acids with separate independent estimations of formic acid and of butyric acid, may be preferred: partition calls for considerable quantities of very pure ether and a critical control of the temperature at which equilibrium is established, whereas the distillation procedure involves nothing other than simple, careful manipulation.

EXPERIMENTAL

A number of 3-acid mixtures containing acetic, propionic and butyric acids, and some 4-acid mixtures containing formic acid in addition, were made up from carefully purified components. Partition constants of the pure acids between water and pure ether at 20° C. were determined; the findings were in accord with the constants reported by Osburn *et al.* (1933).

In the first series of analyses the results from the partition method when applied to 3-acid mixtures containing acetic, propionic and butyric acids, were compared with those derived from solving partition equations after introducing the data obtained from a separate estimation of the concentration of butyric acid by the method of Klinc (1934). The results of analyses of the 4-acid mixtures which contained formic acid are included in this series, the concentration of formic acid in the mixtures being determined by oxidation with mercuric oxide by a procedure slightly modified from that of Osburn *et al.* (1933), and the data so obtained substituted in the partition equations.

Several variables are encountered, among which the actual error of the titrations themselves, the influence on the partition exerted by the concentrations of the individual acids, and changes in the physical conditions under which the partitions are estimated, are probably the main contributors. The observed partitions differed from the theoretical by amounts varying between 0 and 0·5%.

The results from this first series, summarized in Table 1, showed quite definitely that under the conditions of this test, the combined procedure afforded a more accurate analysis when one of the acids was present in relatively high proportion.

In the second series analysed, the results of a distillation procedure were compared with those from the partition method, each being used in conjunction with the

Table 1. *Analysis of mixed fatty acids by two procedures*

Acids present in mixtures (g./l.)				Method used*	Acids found in mixtures (g./l.)				% error in each determination			
Formic	Acetic	Propionic	Butyric		Formic	Acetic	Propionic	Butyric	Formic	Acetic	Propionic	Butyric
—	0·147	0·170	2·16	*a*	—	0·177	0·080	2·22	—	20	53	2·8
				b	—	0·159	0·198	2·15	—	8·2	17	0·5
—	0·147	1·70	0·216	*a*	—	0·159	1·64	0·277	—	8·2	3·5	28
				b	—	0·141	1·71	0·211	—	4·1	0·6	2·3
—	1·47	0·170	0·216	*a*	—	1·51	0·163	0·158	—	2·7	4·1	27
				b	—	1·47	0·159	0·224	—	0	6·5	3·7
—	0·525	0·699	0·898	*a*	—	0·549	0·677	0·893	—	4·6	3·1	0·6
				b	—	0·522	0·722	0·880	—	0·6	3·3	2·0
0·193	1·44	0·229	0·229	*a*	0·189	1·41	0·311	0·185	2·1	2·1	36	19
				b	0·189	1·43	0·252	0·224	2·1	0·7	10	2·2
0·193	0·240	1·83	0·229	*a*	0·193	0·257	1·81	0·229	0	7·1	1·1	0
				b	0·193	0·252	1·82	0·220	0	5·0	0·5	3·9
0·193	0·240	0·229	1·83	*a*	0·198	0·270	0·111	1·92	2·6	13	51	4·9
				b	0·198	0·234	0·255	1·80	2·6	2·5	11	1·6
0·193	0·480	0·459	0·686	*a*	0·198	0·480	0·433	0·717	2·6	0	5·7	4·5
				b	0·198	0·465	0·481	0·682	2·6	3·1	4·8	0·6

* (*a*) Partition method for acetic, propionic and butyric acids. Separate method for formic acid. (*b*) Partition method for acetic and propionic acids. Separate methods for formic and butyric acids.

separate determination of butyric acid. The semi-distillation method was adopted, but only 50 ml. of the mixtures were distilled instead of the 200 ml. recommended by Virtanen & Pulkki (1928). The distillation constants were determined at known concentrations of approximately 0·2 g./100 ml. for each acid. The results which are set out in Table 2 render it clear that the simpler manipulations involved in this distillation may, without loss of accuracy, be employed in place of the more exacting partition procedure.

Table 2. *Comparison between partition and semi-distillation methods*

Acids present in mixtures (g./l.)			Method used*	Acids found in mixtures (g./l.)			% error in each determination		
Acetic	Propionic	Butyric		Acetic	Propionic	Butyric	Acetic	Propionic	Butyric
1·54	0·181	0·261	*a*	1·53	0·194	0·259	0·7	7·2	0·8
			b	1·55	0·170	0·259	0·7	6·1	0·8
0·128	2·17	0·261	*a*	0·120	2·18	0·262	6·2	0·5	0·4
			b	0·134	2·21	0·262	4·7	1·8	0·4
0·128	0·181	3·13	*a*	0·131	0·172	3·13	2·3	5·0	0
			b	0·127	0·176	3·13	0·8	2·8	0

* (*a*) Partition method for acetic and propionic acids. Separate method for butyric acid. (*b*) Semi-distillation method for acetic and propionic acids. Separate method for butyric acid.

SUMMARY

1. The analysis of mixtures of acetic, propionic and butyric acids, in which one of the acids is present in either very large or very small proportions has been examined.

2. A combination of a separate method for butyric acid, with the partition method for acetic and propionic acids, provided more accurate estimations than those obtained directly from the application of the partition method to all three acids.

3. Semi-distillation proved a suitable alternative to partition in the combined procedure.

The author is indebted to Mr H. R. Marston, Chief of the Division, for originally suggesting the problem and for his help and criticism.

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THE DIGESTION OF CELLULOSE BY SHEEP

THE EXTENT OF CELLULOSE DIGESTION AT SUCCESSIVE LEVELS OF THE ALIMENTARY TRACT

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Until recently, information of the extent to which cellulose is digested in the alimentary tract of the ruminant was available only indirectly through studies of the fate of that indefinite and variable complex mixture of cellulose, hemicellulose and encrusting substances which is conventionally referred to as 'crude fibre'. This, in the past, has been taken as a measure of the less readily digested part of the carbohydrates of plants, in contrast to the 'N-free extractives' which represent the more soluble and therefore more readily digestible portion.

Popoff (1875) observed the formation of methane in the rumen as a product of the fermentation of crude fibre; Zuntz described similar fermentative changes in the caecum, and Hofmeister (1880) showed that they also occurred in the colon. Tappeiner (1882) attempted to assess the extent of digestion of crude fibre of the food at different levels as it passed along the alimentary tract of the ruminant. He took samples of the contents of the rumen, small intestine, and caecum from a freshly killed sheep, and estimated the crude fibre which disappeared after 'complete' fermentation at body temperature. From this he concluded that the greater part of the crude fibre was digested in the rumen, a smaller amount in the caecum, and none at all in the small intestine.

A number of workers (Williams & Olmstead, 1935; Norman, 1935; Crampton & Maynard, 1938) have stressed the serious limitations of conventional analyses which divide the carbohydrates of plant foodstuffs into crude fibre and N-free extractives, and have suggested that the analysis of such foods would be more logical and informative if the carbohydrates were divided into better defined groups, such as cellulose, lignin, and other carbohydrates. This proposal has been adopted and extended by Hale, Duncan & Huffman (1940) in their study of digestion in the bovine rumen. These authors developed a method for estimating the extent of digestion in the rumen by the use of 'lignin ratios' and concluded that 85% of the digestible cellulose in alfalfa hay was broken down there.

The following experiments were carried out in this laboratory during 1936. They were mentioned in the Annual Report of this laboratory (Commonwealth of Australia, Council for Scientific and Industrial Research, 1937–38).

METHOD

The lignin ratio procedure was used to determine the extent of cellulose digestion at successive levels of the alimentary tract of the sheep. The method is based essentially on a procedure suggested by Bergeim (1926) for the determination of digestion coefficients. After adding Fe_3O_4 to the food Bergeim determined the ratio of the amount of a given nutrient to the amount of iron in the food and in the faeces, and from this the percentage utilization was calculated. Later Heller, Breedlove & Likely (1928) used the natural iron content of the food as a reference substance, to ensure a more uniform distribution; Gallup (1929) and Gallup & Kuhlman (1931) made use of silica for the same purpose.

The validity of such procedures depends fundamentally on the complete indigestibility of the reference substance, and the exact concurrence of its passage through the alimentary tract with that of the nutrient in question. Knott, Murer & Hodgsen (1936) came to the conclusion that added materials, such as iron oxide, were unsuitable for the calculation of digestion coefficients in ruminants because they were unlikely either to remain evenly distributed in the fodder, or pass along the tract at the same rate as the rest of the food. For a similar reason silica was considered also to be unsuitable; although it is a natural and intimate constituent of plant structures, sand is nearly always present in fodders and may gather in certain parts of the tract rather than move along it at an identical rate with the rest of the food. The absorption of part of the natural iron of the fodder debars its use as a critical reference marker.

Hale *et al.* (1940) have employed lignin as a reference substance for the calculation of digestion coefficients in the rumen. They claimed that it was digestible to a variable extent (up to 24%) in the lower levels of the tract. There have been other claims that lignin is not undigested in various animals including the ruminant (cf. König, 1907; Csonka, Phillips & Jones, 1929; McAnally, 1942), and there are others to the contrary (Paloheimo, 1925; Rogozinski & Starewska, 1927; Crampton & Maynard, 1938). As lignin is not a chemical entity the term, of necessity, has often been used loosely to signify a mixture of similar complexes, some of which may be prone to change in the alimentary tract, and thus may not be expected to react identically towards the various reagents and procedures which different authors have used for its estimation. Since the different methods employed are all empirical, the origin of the controversy is clear enough. Norman (1935) directed attention to this and pointed out that experiments which have depended on the fate of isolated lignin added to the diet are not conclusive since the biological availability of lignin is affected by the processes of isolation.

Lignin was used as a reference marker in the following experiments after it was proved that, as determined in a mixture of wheat straw and lucerne hay by the method of Norman & Jenkins (1934), it was not digested to any appreciable extent by sheep.

EXPERIMENTAL

(1) *The digestion of lignin by sheep*

The evidence that lignin is not digested was drawn from the study of the intake and output of lignin by three sheep fed on a constant diet of wheat straw and lucerne hay for several months while confined to metabolism cages described by Marston (1935). During the fourteen day collection periods the fodder eaten and the faeces voided were measured with meticulous care. Lignin was determined by the method of Norman & Jenkins (1934) in samples from each and the balance drawn up. The data are summarized in Table 1.

Table 1. *Digestion of lignin by sheep*

Sheep no.	Dry wt. fodder eaten (g.)	Dry wt. faeces voided (g.)	Fodder lignin (%)	Faeces lignin (%)	Lignin eaten (g.)	Lignin voided (g.)
W_3A_3	7616	4308	13·84	24·72	1050	1060
W_3A_{43}	7530	4343	12·04	20·91	907	908
W_6 220	7620	4325	12·40	20·92	945	905
W_6 220	7480	4184	12·43	22·00	930	920
W_6 220	8130	4813	12·70	21·01	1030	1010

It may be noted that these results are in agreement with those of Crampton & Maynard (1938) for the digestion of lignin by steers. A different method was used for the determination of lignin, and in which different fodders were fed.

(2) *Extent of cellulose digestion at successive levels of the alimentary tract*

Four sheep which had been fed for some weeks previously on a constant ration composed of wheat straw and lucerne hay were slaughtered, and samples of ingesta were taken from the rumen, omasum, abomasum, small intestine, caecum and colon. The dried, ground samples were subjected to analyses for cellulose and lignin. The method for the determination of lignin has been referred to previously; the method used for the determination of cellulose was that recommended by Norman & Jenkins (1933).

Similar analyses were made of samples of the fodder which the animals had been fed.

Results of these analyses are listed in Table 2.

The data indicate that, of the cellulose present in the fodder, 40–45% was digested before the food passed into the abomasum, and an additional 15–20% was digested in the large intestine. During this second fermentation 7–11% disappeared in the caecum, and 4–9% in the colon. No appreciable loss of cellulose occurred in the abomasum or in the small intestine.

Since the total digestion of cellulose amounted to about 60%, it is apparent that approximately 70% of the cellulose that was digested was broken down in the rumen, 17% in the caecum and 13% in the colon.

Table 2. *Extent of cellulose digestion at successive levels of the alimentary tract*

Level	% lignin	% cellulose	Ratio	% cellulose digested
Fodder	10·41	36·49	3·50	—
Rumen	17·20	35·64	2·07	40·8
Omasum	16·81	35·63	2·12	39·4
Abomasum	13·79	28·40	2·06	41·2
Small intestine	10·90	22·0	2·02	42·3
Caecum	15·12	26·61	1·76	49·7
Colon	18·91	29·01	1·53	56·2
Fodder	12·06	38·61	3·20	—
Rumen	20·71	39·96	1·93	39·7
Omasum	19·07	41·60	2·18	31·9
Abomasum	16·89	32·41	1·92	40·0
Small intestine	13·11	24·50	1·87	41·6
Caecum	20·20	33·11	1·64	48·7
Colon	19·79	27·76	1·40	56·4
Fodder	12·06	38·61	3·20	—
Rumen	21·50	41·09	1·91	40·5
Omasum	21·24	37·72	1·78	44·4
Abomasum	17·35	29·49	1·70	46·8
Small intestine	15·41	25·86	1·68	47·4
Caecum	22·14	29·20	1·32	58·7
Colon	22·30	26·50	1·19	62·8
Fodder	13·94	38·48	2·76	—
Rumen	19·98	34·01	1·70	38·4
Omasum	—	—	—	—
Abomasum	14·21	22·45	1·58	42·8
Small intestine	14·01	22·59	1·61	41·7
Caecum	19·48	26·70	1·37	50·4
Colon	20·76	23·06	1·11	59·8

The claims of Hofmeister (1880) and Tappeiner (1882, 1884) that some digestion of cellulose occurs in the caecum and colon of ruminants were supported by the work of the Dresden school (Ellenberger, 1915–16), although it was stated that the quantity that disappeared in the colon was small. Trautmann & Asher (1939), however, found no fermentation of chemically pure cellulose which had been introduced directly into the caecum and colon of a goat, even after a prolonged sojourn there. They observed no loss of cellulose when rumen contents were introduced into the caecum, and after 24 hr. there, they considered it to be free of rumen flora. These workers claimed that the time ingesta stay in the caecum and colon, together with the fact that there is a rapid water resorption from these organs, must be inhibitive to fermentation processes.

The present experiments, however, support the contention of the earlier investigators that this portion of the tract plays a significant part in the digestion of cellulose by the sheep. Indirect evidence which further supports this view is to be seen in the work of Barcroft, McAnally & Phillipson (1944) who found that volatile fatty acids, the acknowledged products of cellulose fermentation, are present in the blood draining the caecum in considerably greater quantity than in the blood of the peripheral circulation.

SUMMARY

1. Lignin, as determined in these experiments, has been shown to be undigested by sheep.

2. Examination of cellulose-lignin ratios in the fodder, and in material taken from successive levels of the alimentary tracts of sheep indicated that 70% of the digestible cellulose of the fodder was broken down in the rumen, 17% in the caecum, and 13% in the colon. No digestion of cellulose occurred in the abomasum, or in the small intestine.

The author is indebted to Mr H. R. Marston, Chief of the Division, for suggesting the problem, for the specimens from which the lignin balances were determined, and for his help and criticism throughout.

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EXPERIMENTAL MODIFICATION OF THE SEXUAL CYCLE OF THE GREENFINCH

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(With Ten Text-figures)

INTRODUCTION

It is generally assumed that in birds of the northern hemisphere with a brief breeding season sexual maturity is directly related to the seasons. Consequently many attempts have been made to determine the factors which influence the gonads of these birds in spring, and to elucidate the mechanism involved.

Though it has been known in the poultry world for over a century that light has a great influence on reproduction—(artificial light has a stimulating effect on the egg production of hens (Baker & Ranson, 1932) so that an extended length of illumination in autumn protracts the laying period)—Rowan (1926, 1929, 1932, 1938) was the first investigator to demonstrate experimentally the great effect of light on reproductive activities. His experiments were designed to ascertain the relationship between the strong recrudescence of the gonads of migratory birds in spring and the impulse that induces birds to travel north at that time of the year. In this respect Rowan failed, for it has been shown by different authors, e.g. Putzig (1937), Hann (1939) and even by Rowan himself (1932, p. 645) that castrated migrants are able to migrate over long distances.

Starting from the fact that the increase in size of the gonads of migratory birds, the increase in daylight in spring and the northward migration of these birds run parallel, Rowan (1929) submitted juncos (*Junco hyemalis*, a North-American migratory song-bird, belonging to the finches) during midwinter, when their gonads are minute, to increasing amounts of light by means of artificial illumination after sunset. Consequently, the length of day for his experimental birds was increasing in the autumn instead of shortening. Thus, Rowan was able to induce in his birds a rate of gonad recrudescence, exactly comparable to that taking place in spring in control birds. Besides this, his male birds, thus brought into breeding condition, were constantly singing at the end of December, at temperatures between $-20°$ and $-45°$C.; low temperatures have, therefore, no influence on the display of singing.

Post-mortem investigations revealed that the testes of the male juncos had increased enormously in size and that spermatogenesis was at its height. The ovaries of the female juncos had enlarged also under these experimental conditions, but by no means as much as the testes; full-grown follicles did not develop, the diameter of the largest ones measuring only 1·4 mm.

Since Rowan's first detailed publication (1929) his work has been repeated and extended by himself and by many others, among whom Bissonnette and his co-workers especially must be mentioned here. Numerous species of birds and mammals were subjected to increasing dosage of daily illumination, of light of different wave-lengths and of different intensities, and thus brought into oestrus or breeding condition.

Benoit (1935), Ivanova (1935), Ringoen & Kirschbaum (1937) and others experimented with birds which were completely draped in light-proof material or wore silk caps over their heads or had silk caps with slits admitting light freely to the eyes; moreover Benoit (1937) used ducks whose optic nerves were severed or whose eyes were blinded or even removed completely. As in all these cases the testes were activated, it is generally accepted that at least in birds light is the primary factor, causing gonad recrudescence in spring and that the pituitary functions as the intermediary organ between the light impulse and the activation of the gonads. The light stimulus reaches the hypophysis by way of the hypothalamus and activates this endocrine gland to secrete its gonadotrophic hormone, under the influence of which the gonads increase in size and, in case of the testis, sperm formation starts.

At one time in Holland enormous numbers of song-birds were caught in autumn by a traditional method which is sometimes used to-day by local bird-catchers. The song-birds are caught by the use of live male birds as decoys. As it is of primary importance that these decoys should be in full song in autumn, they are gradually put in the dark at the beginning of May and little by little exposed to light again in August, with the result that during September and October they are in full song and then are exceptionally suitable as decoys. This treatment of birds is called to 'muiten' birds; in the course of May the decoy birds are put into the 'muit'* and in August they come out of it. This whole process of 'mewing' was elaborately described by Hoos (1937). Moreover, in Japan, the practice of 'yogai' has been in vogue, which consists in exposing cage-birds to artificial illumination towards the close of the year in order to bring them into singing condition in January.

In connexion with the work of Rowan and others we studied the process of mewing under laboratory conditions, investigation of the state of the gonads during and after mewing being our main concern. In addition we tried to bring these mewed birds again into spring condition by administering additional light during the winter months.

As experimental bird the greenfinch (*Chloris chloris*) was chosen, a hardy species, which remained in good condition throughout the duration of the experiments.

This investigation was suggested by Prof. G. J. van Oordt and carried out under his direction; a preliminary communication about it has already appeared (van Oordt

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* Prof. A. E. H. Swaen has informed me that the English equivalent for the Dutch 'muiten' is to 'mew', i.e. to put a bird into the mew, which was a cage used for hawks, particularly while they were moulting. The word 'mew' will be used in this paper to describe the process of bringing encaged birds gradually into the dark, keeping them in the dark for some time and gradually exposing them to daylight again.

& Damsté, 1939). As a thesis (in Dutch) for the degree of Ph.D. it has been presented in detail to the Faculty of Science of the University of Utrecht.

MATERIAL AND METHODS

Preliminary investigations were begun in the autumn of 1937, when twenty-four greenfinches (13 ♂♂ and 11 ♀♀) were kept in a large aviary, standing in a laboratory room facing south. About half of these birds, which acted as controls, were sacrificed during the winter and spring months in order to investigate their gonads histologically. In the middle of May 1938 six male and six female greenfinches were confined, each in a cage of 28 × 23 × 19 cm. These cages were put into a cupboard, provided with two doors. Tests with a photographic plate showed that when the doors were closed no light entered. Ventilation was arranged through a bent funnel, placed on top of the cupboard.

Before the encaged birds were mewed, they were left in the cupboard with open doors for about a week. During this period they accustomed themselves completely to perches, food-tray and water-bottle. Then mewing was started: the doors were gradually closed, and on 25 May the birds were in complete darkness. Once a week every cage was cleaned and new food and fresh water were given. On 30 July the doors of the cupboard were gradually opened again and on 12 August the birds were once more exposed to full daylight. At regular periods both controls and experimental birds were sacrificed; their gonads were fixed in Bouin's fluid, cut in sections of 5 or 10 μ and stained with haemalum-eosin.

The preliminary investigations had a positive result. When the experimental birds were in the dark their gonads were small and they did not sing, but after they had been exposed to light again they were found to be in breeding condition once more. The experiments were therefore carried out on a larger scale, and as it appeared that for comparative investigations the oval testes were much better suited than the irregularly shaped ovaries, only male birds were used.

In the summers of 1939 and 1940 instead of the cupboard I used a room which could be darkened by means of shutters.

In 1939 the light was decreased from 19 May onwards and on 31 May the birds were in darkness. On 14 August the shutters were reopened and the birds were gradually exposed to daylight; on 21 August they were in full daylight again.

In 1940 fourteen male birds were mewed from 21 May onwards; they were in complete darkness between 30 May and 10 August; on 24 August mewing was finished.

In these three years forty-four male birds in all were mewed.

The quantity of light to which the birds were subjected was measured by means of a luxmeter kindly placed at our disposal by the Physical Laboratory of the University of Utrecht. On 20 May 1939, just before mewing, the quantity of light, measured at a distance of 150 cm. from the window of the room facing north in which the birds were kept, amounted to 900 Lux.

Generally the birds withstood mewing well, but there were some casualties at the beginning of our experiments. In 1939 and 1940 we therefore resorted to the practice of the bird-catchers who give their birds a very small amount of light for 1 hr. per day: we subjected them to a quantity of only 0·006 Lux.

Some of the birds which had been mewed were subjected to further experiments: from the beginning of December four of them received increasing daily amounts of light by means of artificial illumination. The birds were put in a large cage in a room at the south-west side of our laboratory, which was darkened every day by means of curtains from 4.30 p.m. till 8 a.m. (M.E.T.). When the weather was dull, a daylight lamp of 145 W. was kept burning just over the cage; in bright weather the birds received only daylight through a large window from 8 a.m. till 4.30 p.m. From 3 December until 7 January the birds were subjected every day to an increased amount of artificial light, thus having their day lengthened instead of shortened. Owing to technical difficulties this extra illumination was given only in the evening. For instance the birds received:

On 3 December 1940, additional light from 4.30 till 6 p.m.
On 4 December 1940, additional light from 4.30 till 6.10 p.m.
On 5 December 1940, additional light from 4.30 till 6.20 p.m.
On 7 January 1941, additional light from 4.30 till 11 p.m.

From 7 January till 13 February 1941, the same duration of light was maintained.

THE NORMAL REPRODUCTIVE CYCLE

(*a*) *The testis*

It is a well-known fact that the gonads of birds breeding in the temperate and arctic zones are much larger in the breeding season than during the rest of the year. According to Stieve (1921) the testis of the jackdaw (*Colaeus monedula*) in the breeding condition has a volume which is about 1500 × the volume of the quiescent testis; in the house-sparrow (*Passer domesticus*) this factor is about 1100. The minimum size of the testis of the greenfinch is about 0·5 mm. in diameter.

Under laboratory conditions the diameter of the November-December testis of the greenfinch is about 1 mm.; in the breeding season this diameter increases to 8 mm. and more. This considerable enlargement is due partly to the enormous increase in diameter of the testis tubules and partly perhaps to an increased content of fluid, whereas the intertubular spaces are much narrower in spring than in the winter months.

It was found (Table 1) that under laboratory conditions testis activity starts in the first half of February and that the first sperms are formed in the tubules towards the end of March. Sperm formation continues till June; then testis regression begins rather quickly till, in the second half of July, the stage is reached which I have termed for the sake of convenience the quiescent stage and which lasts till the first half of February.

In Table 1 the average diameter of twenty testis tubules is given. From this it follows that the increase in diameter of these tubules coincides with the increase in size of the testis. In Fig. 1 a graph is given, showing the size changes in testes during the course of a year; its maximum occurs during the first half of May.

Table 1. *Showing details of testes of control birds*

No.	Date of autopsy	Average diameter of testis in mm.	Average diameter of testis tubules in μ	Histological details of testes
C 1	5 Jan. 1938	1·3	65	Quiescent stage
C 2	11 Feb. 1939	1·2	110	First beginning of spermatogenesis
C 3	11 Feb. 1939	0·8	60	Quiescent stage
C 4	25 Feb. 1938	2·2	135	Progressive intermed. stage
C 5	10 Mar. 1938	4·–	195	Progressive intermed. stage
C 6	29 Mar. 1938	7·4	355	Complete breeding condition
C 7	31 Mar. 1939	4·4	230	Almost complete breeding condition
C 8	1 May 1939	8·–	340	Complete breeding condition
C 9	19 May 1939	7·6	285	Complete breeding condition
C 10	19 May 1939	6·3	345	Complete breeding condition
C 11	2 June 1939	6·2	410	Complete breeding condition
C 12	7 July 1939	7·1	245	Beginning of regression
C 13	17 July 1939	1·3	70	Almost quiescent stage; much pigment in intertubular tissue
C 14	22 July 1939	1·7	80	Almost quiescent stage; much pigment in intertubular tissue
C 15	28 July 1939	1·1	70	Quiescent stage; intertubular spaces still narrow
C 16	11 Aug. 1939	1·1	60	Quiescent stage
C 17	16 Aug. 1940	2·1	125	Regression stage; intertubular spaces rather narrow
C 18	24 Aug. 1940	1·2	65	Quiescent stage
C 19	16 Oct. 1939	0·5	45	Quiescent stage; much pigment in intertubular tissue
C 20	10 Nov. 1937	1·0	35	Quiescent stage
C 21	8 Dec. 1937	1·1	55	Quiescent stage

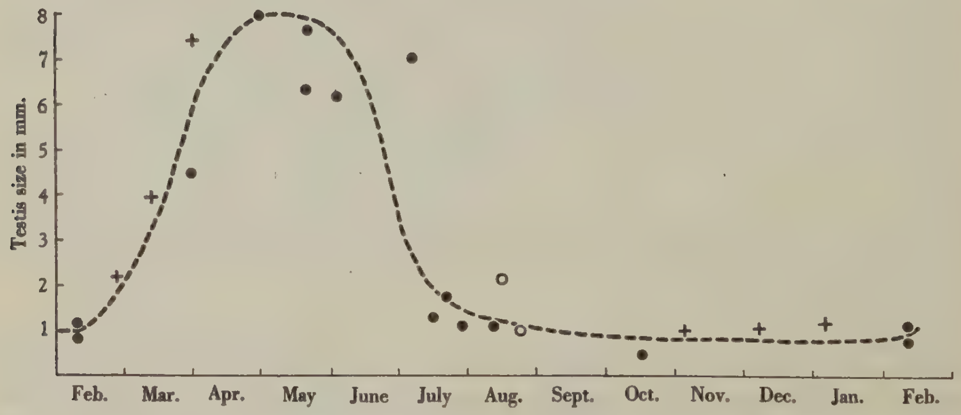

Fig. 1. Graph, showing size changes in control testes in the course of a year. Crosses indicate testes from 1937–38; black dots testes from 1939 and circles testes from 1940.

Approximately the same graph results when the testis tubule diameter is taken into consideration (Fig. 2); its maximum occurs during May.

It is not necessary to give here a full histological description of the gonads of our birds during the course of a year, as an account of the spermatogenesis and a detailed histology of the testis of the greenfinch and of other song-birds has been given elsewhere (e.g. Rowan (1929) for the junco, Bissonnette (1930) and Bissonnette & Chapnick (1930) for the starling). Therefore, only schematic drawings, showing at the same magnification sections of testes during the quiescent stage, the intermediate and the reproductive stage, will be given here.

In Fig. 3 a section of a winter testis of a greenfinch, killed 5 January 1938, is drawn. The section in Fig. 4 represents a testis in the progressive intermediate stage of a greenfinch, killed on 10 March 1938. And finally a cross-section of some tubules of a mature testis belonging to a bird killed 2 June 1939, is shown in Fig. 5.

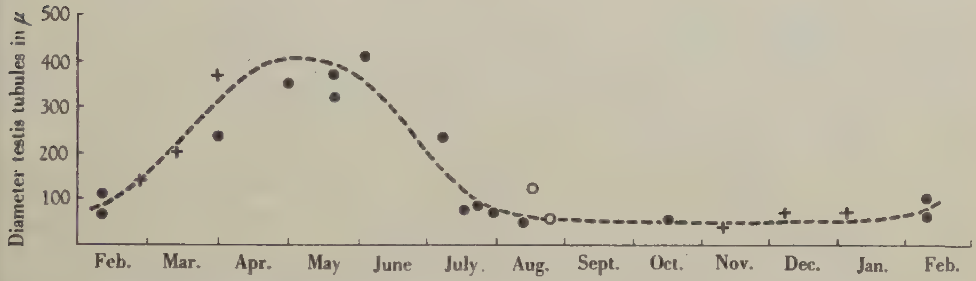

Fig. 2. Graph, showing size changes in testis tubule diameter of normal greenfinches in the course of a year. For explanation of symbols cf. Fig. 1.

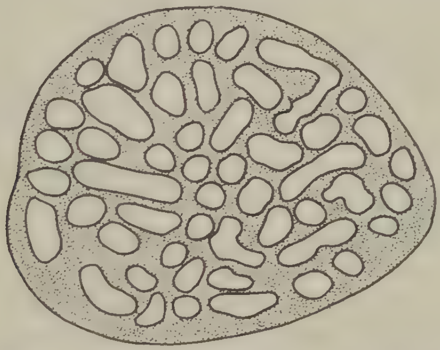

Fig. 3. Contours of testis tubules of a normal greenfinch in the quiescent stage. Intertubular tissue dotted; testis tubules white. C 1, killed 5 January 1938. × 45.

In these figures the relative decrease in size of the intertubular spaces and the enormous increase in diameter of the testis tubules are distinct. Later in the season the quiescent testis stage is approached quickly, in 2 or 3 weeks.

(*b*) *Song*

The male greenfinch has a pretty song, a warbling twitter, in which its characteristic note, a long drawn 'dwee' is often repeated. The song is mostly uttered from a perch, and, in nature, also in flight. In confinement the call note, a short twitter, is regularly heard throughout the year; in early spring the typical 'dwee' is heard first and is soon followed, in March and later, by the true song.

In the laboratory the male control birds, which had started singing from the beginning of March, stopped singing in June.

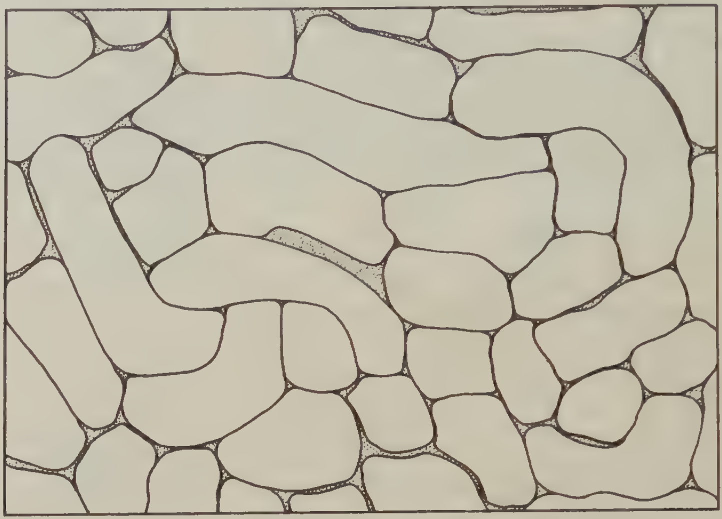

Fig. 4. Contours of testis tubules of a normal greenfinch in the progressive stage. C 5, killed 10 March 1938. × 45.

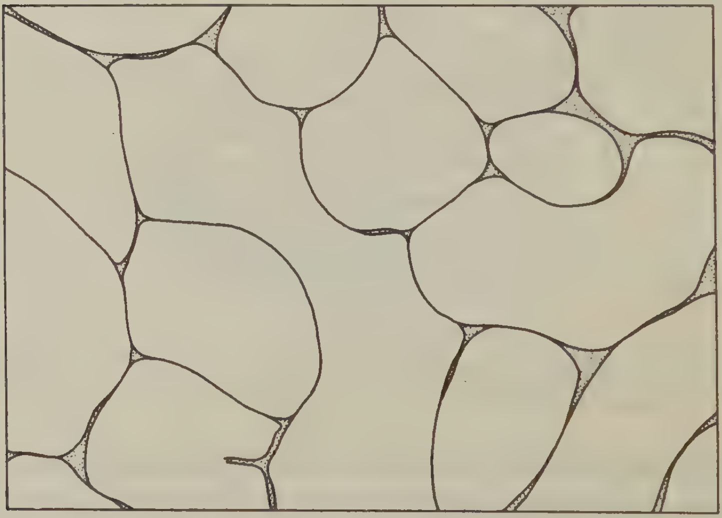

Fig. 5. Contours of testis tubules of a normal greenfinch in breeding condition. C 11, killed 2 June 1939. × 45.

(*c*). *Moult*

The adult greenfinch undergoes a complete moult in nature between the end of July and the beginning of September. In spring there is no moult; but by abrasion of the tips of the feathers the upper parts of the male bird become greener and its under parts brighter yellowish green, while its greater wing coverts and inner secondary flight feathers become greyer and less brown by the same process. The same changes in respect of moult, colour and time of the year were noted under laboratory conditions.

THE EXPERIMENTS

As already mentioned greenfinches were in complete darkness in 1938 from 25 May till 30 July, in 1939 from 31 May till 4 August and in 1940 from 30 May till 10 August. Table 2 gives details of the testes of these experimental birds.

(*a*) *The testis*

The data of Table 2 show that under mewing conditions the testes have involuted completely in 2½–3 weeks (experimental birds E5 and E7) and that they are quiescent during the second half of June and during July. Consequently, the mewed testis is in every way comparable to the winter testis. On the other hand the recrudescence of the testes, induced by light after mewing, is almost as fast as the experimental regression; in experimental birds, which were gradually brought into light in the beginning of August, spermatogenesis is distinct after 1½–2 weeks (birds E24, E25 and E26), whereas sperm formation is complete after 3 weeks (E30 and E32). In this condition the birds remained till about the beginning of October. Then, either owing to the gradually decreasing daylight in autumn or to other inhibiting factors which cause the natural regression of the testis, the gonads diminish in size and towards the end of November or the beginning of December the testes of the experimental birds have reached their winter minimum once more.

Moreover, we have tried to induce a renewed testis recrudescence in these mewed birds. As already mentioned (p. 23) four of our previously mewed birds were subjected in the course of December 1940 and January 1941 to regularly increasing amounts of artificial light, with the result that by the end of December 1940 and in January 1941 the testes of these birds were in full breeding condition, i.e. they had again increased enormously in size and contained large quantities of sperm.

These experiments were concluded at the end of February. It may be accepted that from that time onwards the testes of these birds have shown a renewed regression, possibly due to the relatively shorter days or to some inhibitory influence of the hypophysis. As a matter of fact, at the end of February 1941, the testes of one bird, which had been submitted from 7 January 1941 onwards to artificial light from 4.30 till 11 p.m. and to daylight from about 7.15 a.m. until 4.30 p.m., still showed breeding condition, but at the end of March when only one experimental bird was available, this bird (E44) possessed small testes which were in distinct regression. On that date the bird was exposed to light for about 16 hr.

In Fig. 6 a graph is given, showing the size-changes in the testes of our experimental birds. For the sake of clearness the graph of Fig. 1 is repeated in Fig. 6. In full darkness the testes of the experimental birds are at their minimum, but they enlarge enormously under the influence of daylight during August; in September and the beginning of October they are at their maximum. In November a second minimum is reached, but towards the end of December the testes regain their maximum size under the influence of a newly administered, daily increasing, amount of artificial light.

Table 2. *Showing details of testes of experimental birds.* Nos. E41–E44 *were exposed in December, January and February to 'extra-lighting'*

No.	Date of autopsy	No. of days after beginning of light decrease	No. of days in total darkness	No. of days since beginning of light increase	No. of days since end of mewing	Average diameter of testes in mm.	Average diameter of 20 testis tubules in μ	Histological details
E 1	19 May 1939	0	—	—	—	6·7	260	Breeding condition
E 2	21 May 1940	0	—	—	—	8·—	440	Breeding condition
E 3	26 May 1939	7	—	—	—	5·1	260	Breeding condition
E 4	2 June 1939	14	2	—	—	6·2	440	Breeding condition
E 5	4 June 1940	14	5	—	—	5·2	355	Beginning of regression
E 6	9 June 1939	21	9	—	—	6·6	440	Breeding condition
E 7	15 June 1938	35	27	—	—	1·3	75	Quiescent stage
E 8	16 June 1939	28	16	—	—	1·7	90	Advanced regression stage
E 9	17 June 1939	29	17	—	—	5·5	290	Regression stage
E 10	23 June 1939	35	23	—	—	2·1	130	Advanced regression stage
E 11	28 June 1939	40	28	—	—	1·9	100	Almost quiescent stage
E 12	28 June 1939	40	28	—	—	2·4	140	Almost quiescent stage
E 13	3 July 1939	45	33	—	—	1·—	75	Quiescent stage
E 14	7 July 1939	49	37	—	—	2·—	230	Strong regression
E 15	14 July 1939	56	44	—	—	3·3	140	Regression stage
E 16	15 July 1938	65	57	—	—	3·2	280	Weak regression
E 17	21 July 1939	63	51	—	—	1·6	100	Almost quiescent stage
E 18	22 July 1939	64	52	—	—	1·5	65	Quiescent stage
E 19	28 July 1939	70	58	—	—	1·1	75	Quiescent stage
E 20	30 July 1940	70	61	—	—	1·2	65	Quiescent stage
E 21	8 Aug. 1939	81	69	4	—	1·7	110	Strong regression
E 22	10 Aug. 1940	81	71	0	—	1·2	60	Quiescent stage
E 23	12 Aug. 1938	93	85	13	0	1·6	105	Almost quiescent stage
E 24	15 Aug. 1938	—	—	16	3	5·6	340	Progressive stage
E 25	15 Aug. 1938	—	—	16	3	1·8	125	Beginning of spermatogenesis
E 26	16 Aug. 1939	—	—	12	—	2·9	290	Progressive stage
E 27	24 Aug. 1940	—	—	14	0	3·7	245	Breeding condition
E 28	25 Aug. 1939	—	—	21	4	3·8	320	Almost breeding condition
E 29	25 Aug. 1939	—	—	21	4	3·7	355	Almost breeding condition
E 30	30 Aug. 1938	—	—	31	18	4·7	315	Breeding condition
E 31	31 Aug. 1940	—	—	21	7	3·5	205	Progressive stage
E 32	8 Sept. 1939	—	—	35	18	6·0	415	Breeding condition
E 33	14 Sept. 1940	—	—	35	21	6·3	435	Breeding condition
E 34	25 Sept. 1939	—	—	52	35	7·5	305	Breeding condition
E 35	5 Oct. 1940	—	—	56	42	6·7	510	Breeding condition
E 36	1 Nov. 1939	—	—	88	71	4·0	290	Breeding condition
E 37	4 Nov. 1940	—	—	86	72	3·—	265	Beginning of regression
E 38	30 Nov. 1940	—	—	112	98	2·1	125	Regression stage
E 39	7 Dec. 1939	—	—	125	108	3·9	255	Beginning of regression
E 40	11 Dec. 1939	—	—	129	112	1·5	85	Quiescent stage
E 41	30 Dec. 1940	—	—	27	—	5·3	390	Breeding condition
E 22	28 Jan. 1941	—	—	56	—	6·9	470	Breeding condition

In the experimental birds the testis tubule diameter runs parallel to the total testis size, which is clearly perceptible in Fig. 7, showing a graph of the different testis tubule diameters throughout the experimental period.

As it may be accepted that these birds were also in breeding condition in the spring of the same year, it is obvious that the experimental birds were mature three times within a year: first, in April and May before they were mewed; secondly in August and September after having been mewed; and thirdly, in December and January when their testes underwent a third recrudescence under the influence of administered artificial light.

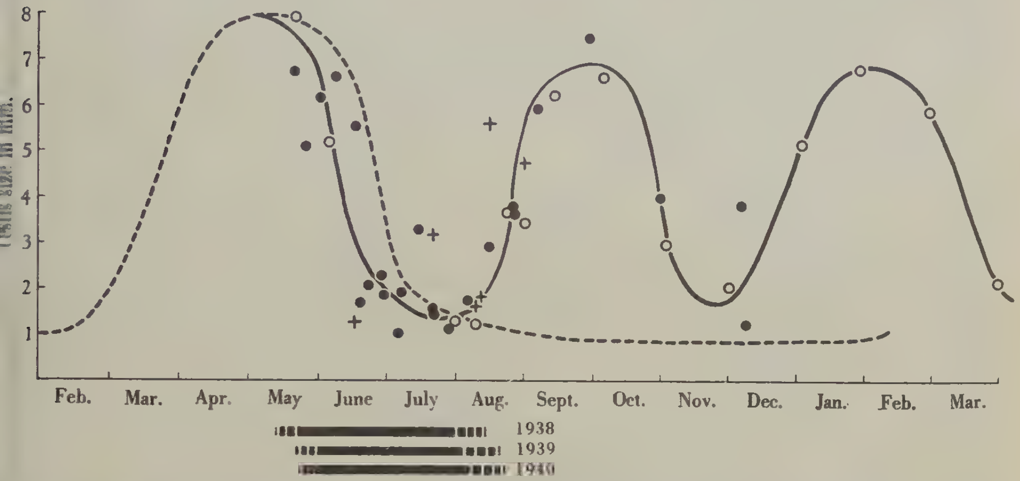

Fig. 6. Graph, showing size changes in testes of experimental birds. The periods of mewing in 1938, 1939 and 1940 respectively are indicated by heavy black lines at the bottom of the figure. The broken line relates to the size changes of control testes. For explanation of symbols, cf. Fig. 1.

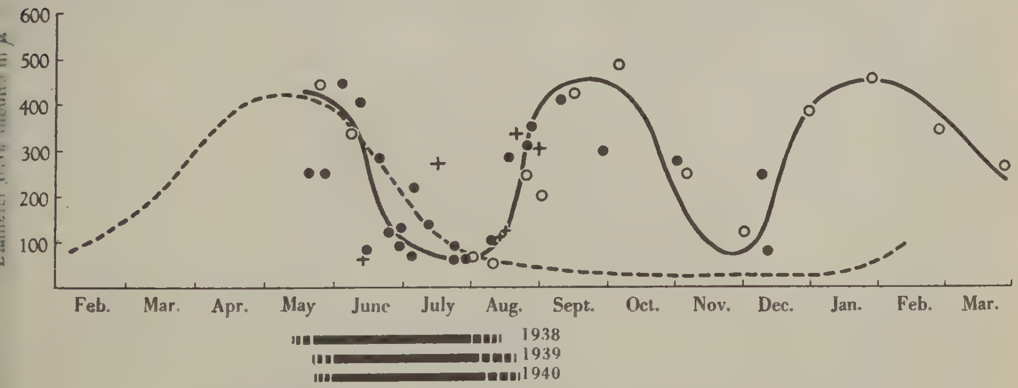

Fig. 7. Graph, showing average testis tubule diameter of experimental birds. The broken line relates to the size changes of testis tubules of control birds (cf. Fig. 2). For further explanation, cf. Figs. 1 and 6.

I think it superfluous to reproduce here many sections of the testes of the experimental birds with histological details, as the latter are exactly the same as in the control testes. The experimental testes are normal in all respects, except as to dates. Fig. 8 shows a part of a section of a testis in regression; it belonged to a greenfinch which had been in total darkness for only 5 days. Fig. 9 represents a drawing of the section of a testis of a greenfinch in the quiescent stage during mewing. In Fig. 10 histological details of the same testis are shown.

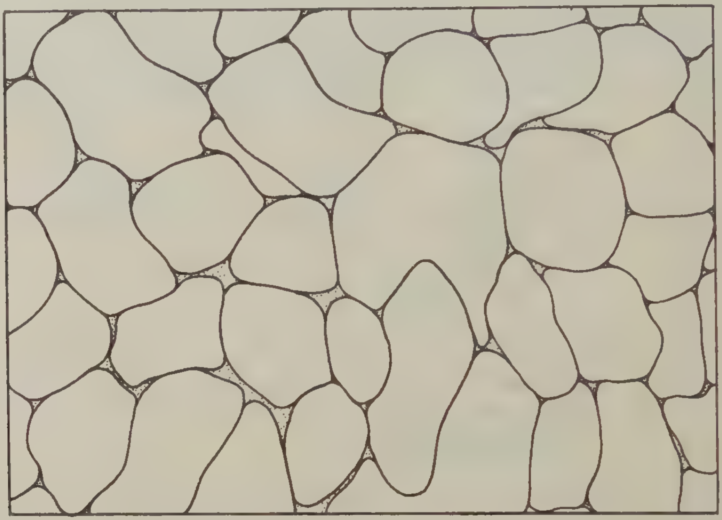

Fig. 8. Contours of regressive testis tubules of an experimental greenfinch during mewing. E 5, killed 4 June 1940. ×45.

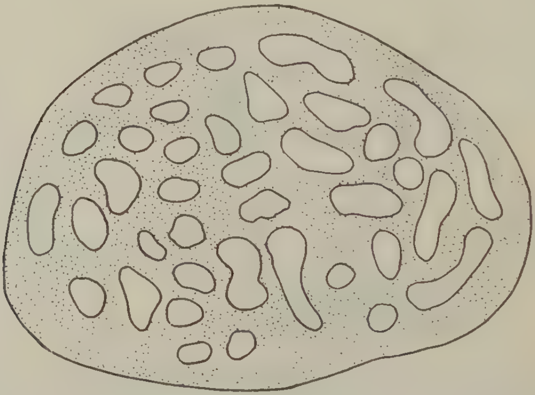

Fig. 9. Contours of testis tubules of an experimental greenfinch during mewing; quiescent stage. E 7, killed 15 June 1938. ×45.

(*b*) *Song*

Very soon after the birds were exposed to a decreased quantity of light they ceased singing; only the characteristic 'dwee' and an occasional twitter were heard, but as soon as the birds were kept in continual darkness they were quite silent. With the regression of the testes the song had come to an end.

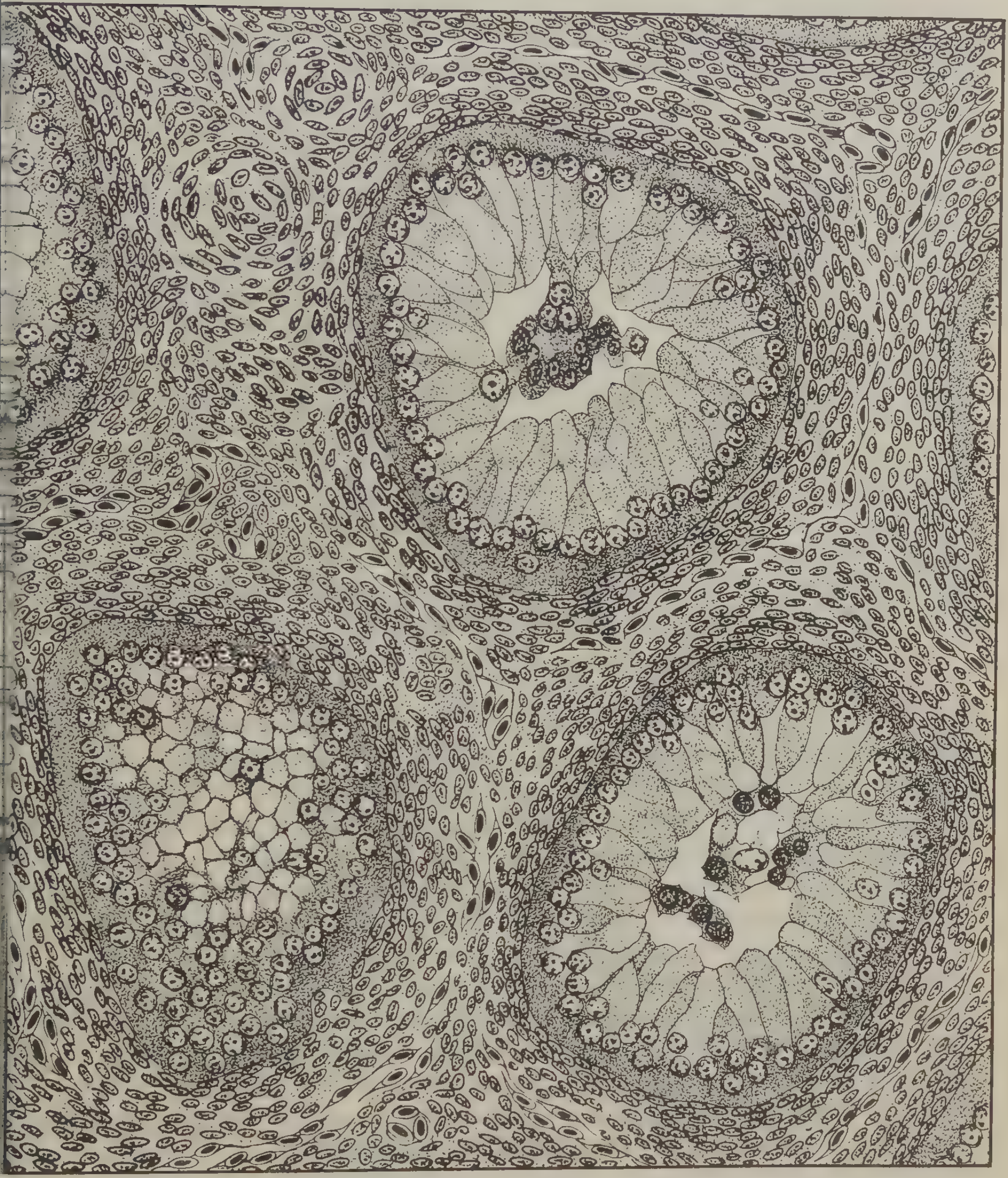

Fig. 10. Histological details of part of the section of the testis shown in Fig. 9. E 7, killed 15 June 1938. × 600.

When at the end of the mewing period light was once again administered, the twitter was heard again, soon followed by the 'dwee'-call and at the end of August and in September the experimental birds were in full song. The same thing was observed in birds brought into breeding condition by artificial light in December. Therefore recrudescence of the testes coincides with initiation of singing; when the testes are in breeding condition the song is also at its height.

(*c*) *Moult*

It was observed that during mewing the birds were not only moulting, but also that moulting and refeathering started much earlier than ordinarily. As mentioned on p. 26 the summer moult and refeathering take place under normal conditions in August. In our experimental birds, on the contrary, moulting and refeathering started as early as the first half of June when they were in utter darkness. Then many coverts were shed and shortly after not only the flight- and tail-feathers but also the down followed. In the experimental birds moulting was at its maximum at the end of June. Then the feathers reappeared gradually and by the end of July refeathering was completed in most of the birds. Therefore, it is possible that a relation exists between the effect of darkness and the processes of moulting and refeathering. However, from our observations, it could not be ascertained whether this precocious moult might not be due to some other cause, e.g. the state of the gonads or the state of the thyroids, which, as one knows, play an important part in the process of moulting. We shall consider this subject in the discussion (p. 34).

DISCUSSION

From the above it follows that as a consequence of mewing the testes of the experimental birds were minute and that after mewing the birds developed testes with large quantities of sperm and were in full song. Moreover, some of these birds, having been exposed to increasing quantities of (electric) light towards the close of the year develop fertile testes and at the same time come again into full song. So it was possible by means of mewing and 'extra lighting', within the course of a year, to bring greenfinches twice into breeding condition after the normal breeding season in April and May.

In the light of Rowan's and Bissonnette's investigations it is easy to understand that after an involution stage, the result of bringing the birds into darkness, their testes enlarge rapidly till, under the influence of the increasing daylight, they are fully mature after some weeks of increasing spermatogenesis. Then, in August, the birds are in full song. So it is likely that song coincides with sexual maturity and is induced by the male sex hormone, this being in agreement with the experiments of Leonard (1939) and of Frederiks (1941) who were able to induce singing by means of androgen administration in female canaries, and with the fact that castrated male birds do not sing.

Most probably, due to the shortening days in October, testis regression starts again in the course of this month, followed by a stage of sexual inactivity, which coincides with a non-singing period. Exposure of these birds to increasing quantities of (electric) light from the beginning of December till the beginning of February again induces full spermatogenesis and full song.

According to Rowan (vide Rowan, 1938, for a summary) the influence of light on the testis, which results in sperm formation, is not direct but indirect, as the light merely permits the birds to remain awake, and this wakefulness causes an increased physical activity, which is the primary sexually activating stimulus. Rowan's hypothesis has not been confirmed by the experiments of many other investigators (Bissonnette, 1931, 1933, 1936; Riley, 1937; Kendeigh, 1941) who found that enforced exercise in darkness did not induce testicular activation in starlings or house-sparrows. Neither are the experiments of Benoit (1937) who tied up his ducks nor those of Miyazaki (1934, 1935) who observed that during 'yogai' (cf. p. 21) his birds (*Zosterops palpebrosa japonica*) were sleepy and inactive, in favour of Rowan's opinion. Moreover, my own experiments do not support his view; my greenfinches were placed in small cages, in which they could only move a little; nevertheless, when they were exposed to light again, their testes were activated very quickly.

Rowan (1929) and Miyazaki (1934) also succeeded in bringing birds into the breeding condition more than once within the course of a year. Rowan treated juncos with extra light during the autumn, then to shortened days in January and finally to the increasing daylight of February and March. By the end of May these birds were found to be in full breeding condition once more. Miyazaki practised 'yogai' with his birds during December, and got singing birds in January; then a second 'yogai' was initiated during September and stopped at the end of October when the birds were again in full song; and after a third 'yogai', started at the beginning of December, the birds reached breeding condition again towards the end of the year.

As to the time of year in which song-birds may be brought into breeding condition, the opinions of the authors differ. Riley (1937) was not able to activate the testes of house-sparrows in September, except in juvenile birds; the same negative result was obtained by Schildmacher (1938, 1939) with redstarts (*Phoenicurus phoenicurus*), robins (*Erithacus rubecula*) and blackcaps (*Sylvia atricapilla*) in the early months of autumn. My own experiments showed, however, that testis recrudescence may occur already in August after the birds have been mewed.

Some investigators (e.g. Benoit (1937) and Bissonnette (1938)) have found that after a period of extra lighting the enlarged testes regress spontaneously. This phenomenon was also observed during my experiments in a bird (E44), which had been in breeding condition three times within 12 months and in which testis regression occurred, although it was subjected to increasing lengths of daylight during March. The origin of this regression is not clear; internal factors may play an important part.

As to the path along which the light stimulus is transmitted to the testes, it is generally accepted that it reaches the hypophysis by way of the eyes, optic nerves and hypothalamus, and activates this endocrine gland to secrete its gonadotrophic hormone, under the influence of which the gonad recrudescence and sperm production take place.

It would therefore have been important if it had been possible to detect in the anterior lobe of the hypophysis, simultaneously with the gonad recrudescence, an increase in number of the so-called basophile cells, to which the gonadotrophic function of the hypophysis is generally attributed. However, I was not able to establish a distinct difference between the acidophile (the α-) and the basophile (the β-) cells with the aid of the usual colour methods ('Azan'); even 'Duazor' (Ruyter, 1943) gave no results. Therefore it was not possible to discern a parallel between the gonad activity and an increased activity or a larger number of β-cells in the hypophysis. As far as I am aware the avian hypophysis stains very poorly, a fact which has also been established by other investigators in our laboratory; so it was impossible to make a qualitative or a quantitative cytological study of the anterior lobe of the hypophysis.

A precocious moult and refeathering were observed in the birds subjected to mewing. In nature the summer moult of the adult greenfinch takes place between the end of July and the beginning of September. Under the experimental conditions, described above, moulting and refeathering occur very much earlier; the reduction of light causes these phenomena to take place in the first half of June. Burger (1941) found in starlings that a reduction in length of day favours or speeds up moulting and refeathering. Moreover, Miyazaki (1934) observed a moult in his birds (*Zosterops palpebrosa japonica*) subjected to 'yogai', after which the birds received as a matter of course relatively less light than under 'yogai' conditions. Walton (1937) caused mallard drakes to take an eclipse plumage during February and March instead of during June and July by subjecting them to extra lighting.

Moulting and refeathering, being the expression of processes of metabolism, are undoubtedly related to the hormonal function of the thyroid; therefore it seems important to study the histology and cytology of this endocrine gland in order to establish a possible relation between the microscopical structure of the thyroid and the precocious moult. As the function of the thyroid is in turn regulated by the anterior lobe of the hypophysis it would also be important to study the anterior lobe, as the acidophile cells are possibly the source of the thyrotrophic hormone. Preliminary investigations on this subject have been carried out already, but will not be published till more experimental material is available.

My experiments as well as those of numerous other investigators have shown that in the male song-bird light is an essential factor in producing testis recrudescence in spring. Of course other factors, amongst which are the reciprocal stimulation of the males and the presence of the female, certainly have an important role in testis activation. In the female, however, these factors are more important and more numerous, as has been shown by the experiments of Polikarpova (1940) with female sparrows, where ovulation occurs only under special circumstances.

SUMMARY

1. 'Mewing' of song-birds is a traditional practice of Dutch bird-catchers who make use of it to produce singing decoys in the autumn. The birds are encaged early in May, the daylight is progressively reduced by means of curtains during May and

the birds are kept in darkness throughout June, July and the first half of August. From the middle of August onwards the curtains are gradually opened and by the end of the month the birds are in daylight and in full song once more.

2. This procedure was carried out with the greenfinch (*Chloris chloris*) under laboratory conditions in order to study the behaviour, song and moulting of the birds and the histology of their gonads before, during and after mewing.

3. In the early stages of mewing the testes decrease in size till in total darkness they have the same diameter as the inactive testis.

4. When the birds are exposed to normal daylight again the testes enlarge rapidly until after some weeks of increasing spermatogenesis they are fully mature. By this time the birds are in full song and it is likely that song coincides with sexual maturity and is induced by the male sex hormone.

5. The diameter of the testis tubules varies in the same way as the diameter of the gonads.

6. In the middle of October, after some weeks of sperm production, regression of the testis sets in. During the ensuing stage of sexual inactivity some birds were exposed to increasing quantities of (electric) light from the beginning of December until the beginning of February. These birds showed increasing spermatogenesis and developed their full song.

7. Thus by means of mewing and 'extra lighting' it was possible to bring birds into breeding condition three times within one year.

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THE EFFECT OF LABYRINTHECTOMY ON THE CO-ORDINATION OF LIMB MOVEMENTS IN THE TOAD

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(With Plate 1)

If the limbs of an animal are to propel the body along a straight horizontal line the propulsive forces exerted by the right and left pairs of limbs must be equal and the vertical thrusts exerted by each of the four limbs must be such that their combined resultant is equal to the weight of the body and act through its centre of gravity (Gray, 1944). During the normal ambulation of a toad these conditions are not strictly fulfilled, for the body exhibits a slight but definite tendency to rotate about its principal axes (Barclay, 1946). Since all such movements are liable to induce compensatory reflexes of labyrinthine origin, it is of interest to know how far the normal level of co-ordination between the limbs is affected by labyrinthectomy. In all cases the operation has been performed from the dorsal side.

THE EFFECT OF LABYRINTHECTOMY ON AMBULATION

After bilateral labyrinthectomy a toad exhibits relatively little spontaneous movements on land and tends to remain at rest with its hindlimbs partially extended and its head depressed towards the ground. If, however, sharp pressure be applied simultaneously to the plantae of both hindfeet the animal frequently responds by ambulation, the progression of the animal being of the normal type. The limbs are protracted in diagonal sequence and the animal progresses along a rectilinear path. It is therefore clear that the labyrinths do not play a fundamental role in the maintenance of the normal diagonal rhythm of limb movements or in the ability of the limbs to support and propel the body; in the absence of the labyrinths, the co-ordination of the thrusts exerted by the limbs and the sequence of movements of the limbs are controlled by the proprioceptor mechanisms described in previous papers. In the intact animal, however, proprioceptor and labyrinthine responses are very closely associated with each other. This fact is well illustrated when a labyrinthectomized animal is set in motion in response to a tactile stimulus applied to one side of the body; in this case, the resultant ambulation takes place along a path which constantly curves towards the unstimulated side. If the stimulus be weak the radius of the track is usually large and the limbs retain their diagonal sequence of movement; the limbs on the stimulated side take longer steps than those on the unstimulated side. If, however, the exciting stimulus is relatively intense, the head turns sharply towards the unstimulated side of the body and the animal proceeds to 'circle' actively without making any effective progress over the ground. The limbs on the stimulated side step forward in alternating sequence, whilst those on the unstimulated side step backward. The sequence in which the limbs are lifted from the ground is no longer diagonal but rotary (*RF*, *LF*, *LH*, *RH*). The physiological basis of this circling movement is by no means clear, but it is dependent on the integrity of the sensory nerves of the limbs. No circling, or other locomotory, movements ever occur if all the spinal nerves of the labyrinthectomized animal are de-afferentated.*

After complete spinal de-afferentation a sharp stimulus applied to the right jaw of a labyrinthectomized animal elicits a typical monophasic response; the right forelimb is adducted, the right hindlimb is extended (with or without abduction) and the left hindlimb flexes. All these responses tend to move the animal away from the source of the stimulus. A similar stimulus applied to the jaw of a labyrinthectomized animal, whose sensory nerves are intact, elicits circling movements, whereas in the intact animal the initial turn towards one side is followed by rectilinear progression. Pending further experimental data it would appear that the response of the intact animal to a relatively violent stimulus applied to one side of its body consists of three components: (i) A monophasic response independent of both proprioceptors and labyrinths. (ii) A response dependent on the proprioceptors which, when unaffected by the labyrinths, tends to reproduce rhythmically the same mechanical effects as the first monophasic response. (iii) A compensating response due to the labyrinths which enables the proprioceptor rhythm to be transferred from the muscles involved by the initial response to those concerned with rectilinear progression.

The close association between proprioceptor and labyrinthine excitation is also illustrated by the effect of labyrinthectomy on the ability of a toad to use a de-afferentated limb for ambulatory purposes. In the absence of the labyrinths a de-afferentated limb frequently fails to exhibit any ambulatory movements, progression being effected entirely by the intact limbs.

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* The 'circling' response to a strong unilateral stimulus is remarkably persistent in the sense that it continues for relatively long periods after the cessation of the stimulus. It is also remarkable in another respect: a stimulus applied to one side causes persistent circling which eventually ceases; if shortly afterwards a second stimulus is applied to the opposite side the direction of circling is not reversed but is resumed in the original direction. Only after a prolonged period of rest or after a period of 'hypnosis' does the direction of circling invariably occur towards the unstimulated side of the animal. The significance of these phenomena is obscure. Circling does not occur in a unilaterally labyrinthectomized animal. If the animal is gently pressed between two glass plates it can be turned with its dorsal side uppermost, without (in the case of the labyrinthectomized animal) exhibiting any tendency to struggle. The upper plate can now be very slowly raised until out of contact with the animal which then lies motionless on its back. In this position the toad may remain motionless for as much as 5 or 10 min., after which it rights itself spontaneously; if, however, during the 'hypnotic' state the toad is very gently stimulated it immediately rights itself. A similar phenomenon can be observed in intact animals after steady compression, in the inverted condition, between the palms of the hand.

THE EFFECT OF LABYRINTHECTOMY ON SWIMMING

If the labyrinth be removed from one side of an otherwise intact toad the phasing and frequency of the propulsive swimming strokes remains unaffected, but, sometimes, the amplitude and power of the limb on the operated side are reduced and the animal swims towards that side. If both labyrinths are removed all effective co-ordination between the two hindlimbs is lost and the motion of the body in the water becomes completely unco-ordinated; the frequency of the movements of the two hindlimbs not infrequently appears to remain the same, but the power of adjusting the strength and direction of thrust on the two sides of the body is absent.

As previously reported (Gray & Lissmann, 1946) a toad can swim after severance of the dorsal roots of all its spinal nerves; such preparations usually fatigue rapidly, but the level of co-ordinated movement is fully maintained so long as movement persists. On the other hand, all swimming movements are permanently lost if total spinal de-afferentation is accompanied by bilateral labyrinthectomy. If only one labyrinth be removed, the ipsilateral de-afferentated limb frequently fails to exhibit swimming movements, whereas the contralateral limb on the intact side remains active. These facts suggest that the maintenance of a well-sustained and well co-ordinated swimming rhythm is dependent on the integrity of the membranous labyrinths and of the proprioceptor mechanism of the limbs themselves. In the absence of the proprioceptor components of stimulation, the labyrinths alone can excite and control the motor centres of the limb musculature but cannot sustain a rhythm of activity for very long; in the absence of labyrinthine stimulation, the proprioceptors of a limb can sustain active swimming movements but cannot maintain co-ordinated movements between the two hindlimbs.

How far the swimming rhythm of a spinally de-afferentated animal is due to a rhythm of stimulation emerging from the labyrinths is uncertain, but the following experiment suggests that this may be the case. The hind region of a toad was mechanically isolated from the head by dislocation of the ilio-sacral articulations, and severance of all structures except the nerves and main blood vessels uniting the anterior and posterior regions of the preparation. The head and denervated body were then mounted on a cork and the preparation floated in Ringer solution; swimming movements of the hindlegs were readily elicitable so long as the cork was free to move, but as soon as the cork was rigidly fixed all swimming movements ceased. This suggests that, during normal swimming, the acceleration applied to the body by the hindlimbs excites the labyrinths which then reflexly induce movement of the hindlimbs. We have also frequently observed that swimming movements can readily be induced in totally de-afferentated animals by accelerating them quickly over a smooth surface.

THE EFFECT OF LABYRINTHECTOMY ON THE RIGHTING REACTION

Perhaps the most striking effect of bilateral labyrinthectomy on the toad is the failure of this operation to abolish the power of the animal to right itself when forcibly placed on its back. This confirms the observations of Beritoff (1927) on the frog.

The mechanics of the righting response in the operated animal are fundamentally the same as those of the intact animal although the process is usually slower. The mechanism, as determined by cinematograph records, is (for an animal righting itself by rolling towards its right side) as follows: (i) The left hindleg flexes and lies in contact with the ground, the plantar surface being held in a more or less vertical plane. The right limb is fully extended, and retracted, the posture of the forelimbs being variable but never fully flexed. (ii) The righting of the body is effected by the rotation and extension of the left hindlimb. Rotation of the limb's axis towards the ventral surface of the body raises the left hip, at the same time bringing the plantar surface into contact with the ground. In this way the mechanical axis of the left hindlimb passes well above the right side of the body and the left plantar surface forms a firm *point d'appui.* (iii) The extensor muscles of the left knee and ankle extend, thus raising and pushing the left hip towards the right side. In this way the centre of gravity is eventually displaced beyond and to the right of the bearing surface of the body. From this point onwards the body falls under its own weight—the fall being broken by the extended left forelimb. The process of righting is shown in Pl. 1. Beritoff concluded that the righting response of the labyrinthectomized frog is of proprioceptor origin, since removal of the skin and various other organs did not abolish the response. With this view we agree. A toad can right itself either by response to proprioceptor or to labyrinthine excitation, and under normal conditions the two sources of stimulation reinforce one another. The general relationship between the proprioceptor and the labyrinth is illustrated by the following experiment in which the proprioceptor organs of the hind end of the body can be made to operate with or against the labyrinths. Under ether anaesthesia, the body and forelimbs of an animal were completely denervated, the ilia were detached from the vertebral column, a circular transection was made to the body wall and skin, and the gut was removed. In this way the posterior limbs could be rotated independently of the anterior region of the body. The preparation was then allowed to recover from the anaesthetic. If the whole preparation was then placed on its back, the hindquarters immediately righted themselves and thereafter remained at rest. If the whole preparation were resting on its ventral surface, a sharp lateral tilt of the anterior region towards the right elicited only an extension of the right knee; if, on the other hand, a similar tilt was applied when the anterior region was on its ventral surface and the posterior on its back, the response of the ipsilateral hindlimb was much more active and the hindquarters righted themselves. These observations suggest that when a normal animal is placed on its back the righting response is due to a mutual reinforcement of labyrinthine and proprioceptor stimulation, either of which may be adequate for the righting of the body; under artificial conditions, however, the labyrinths may work with or against the proprioceptors.

SUMMARY

1. After bilateral labyrinthectomy, a toad can only walk normally if the stimulus which excites it to move is applied symmetrically on the two sides of the body. If the stimulus is asymmetrical, the animal's path curves towards the unstimulated

side; if the stimulus is relatively intense the animal 'circles' persistently towards the unstimulated side. The ambulatory response of a normal animal to an asymmetrical stimulus is dependent on both proprioceptive and labyrinthine activity.

2. After bilateral labyrinthectomy, both hindlimbs exhibit swimming movements when the animal is freely suspended in water but co-ordination between the two limbs is lost. Evidence is put forward which suggests that the swimming rhythm of the limbs may be dependent on rhythmical excitation of the membranous labyrinth. No swimming occurs when the limbs of a labyrinthectomized toad are de-afferentated.

3. The removal of both labyrinths does not abolish the power of the limbs to right the animal when placed on its back. The mechanics of the righting movement are described.

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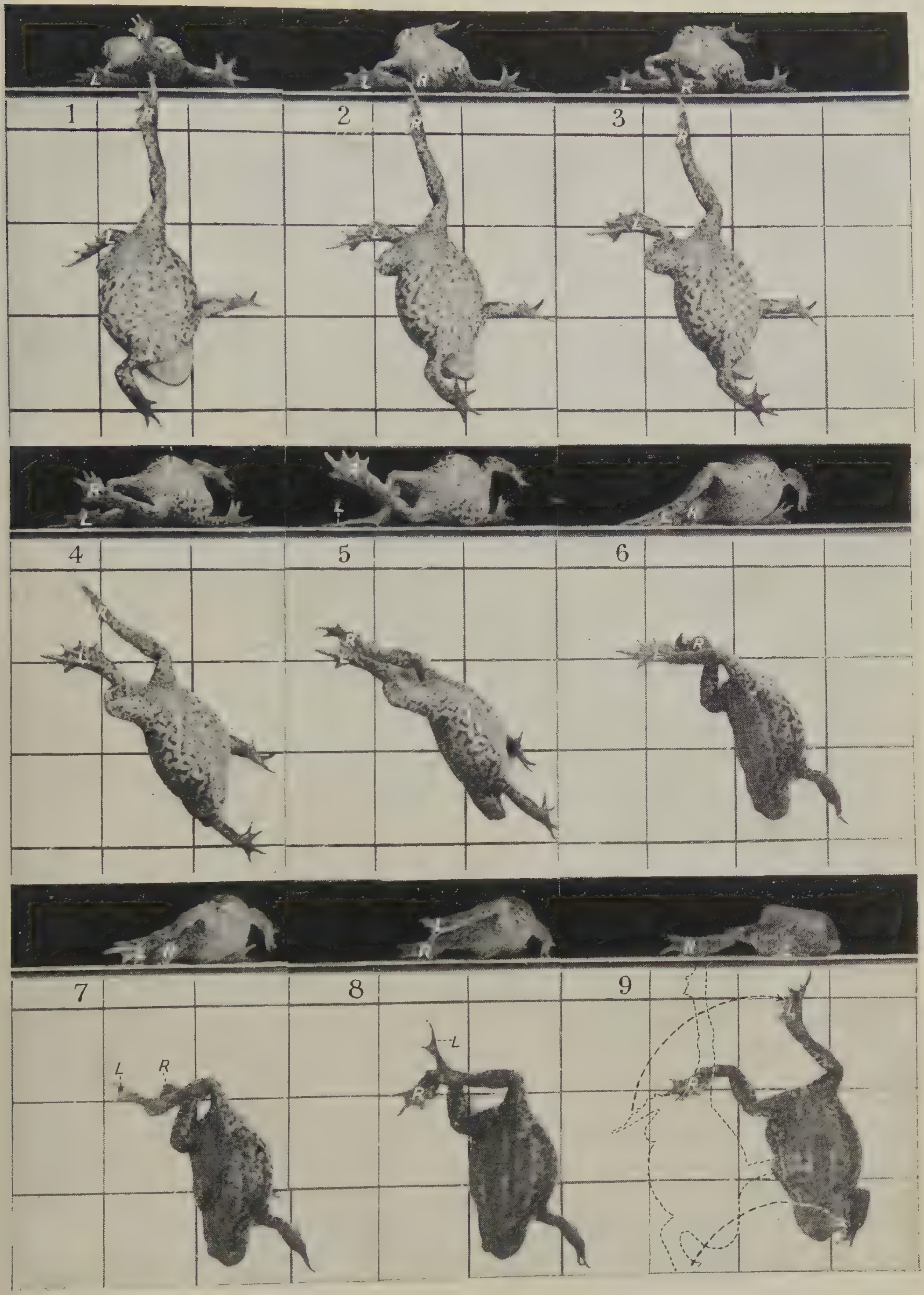
EXPLANATION OF PLATE 1

Successive cinematograph pictures of the righting response of a normal toad; each figure shows a vertical view and a posterior horizontal view, the latter as seen in a mirror.

In phot. 1 the left hindlimb is flexed, the foot being in contact with the ground along its post-axial border. In phots. 2–5 the axis of the left hindlimb has rotated relatively to the body, thus lifting the left hip and bringing the whole plantar surface into contact with the ground. At the same time the left hindlimb extends and pushes the centre of gravity of the body over the surface of support which is provided by the right side of the body. From this point onwards (phot. 6) the latter falls under its own weight on to its ventral surface—the impact being broken by the right forelimb. *L* and *R*, left and right hindlimb respectively. In phot. 9 the original position of the animal as seen in phot. 1 is indicated by the outline figure.



GRAY AND LISSMAN—THE EFFECT OF LABYRINTHECTOMY ON THE CO-ORDINATION OF LIMB MOVEMENTS IN THE TOAD

PRELIMINARY STUDIES ON THE NUTRITIONAL REQUIREMENTS OF THE BEDBUG (*CIMEX LECTULARIUS* L.) AND THE TICK *ORNITHODORUS MOUBATA* MURRAY

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(With One Text-figure)

INTRODUCTION

The nutritional requirements of insects have been the object of much investigation in recent years. These studies have been largely confined to holometabolous insects which feed on a variety of unsterile food in nature. By working under aseptic conditions in the laboratory the exact requirements of the insects concerned may be assessed. The original object of these investigations was to determine the nutritional requirements of a hemimetabolous insect, *Cimex lectularius*, which during its whole life takes only sterile blood as a source of food. For this purpose it became necessary to feed the insects *in vitro* through a membrane so that the medium presented to them could be varied at will. It was also intended to induce changes in the blood taken by the bugs *in vivo*, i.e. in the living animal on which they were fed.

It was soon discovered that the work presented rather formidable technical difficulties. Since the bedbug is a selective feeder it is necessary to pander to its tastes, and nothing can be done to overcome its refusal to take nutritive media presented to it. No doubt these difficulties will be resolved in time. It also became obvious at an early stage that while *in vitro* animal blood could easily be rendered unsuitable for bedbugs, it was very difficult to do so *in vivo*. *In vitro* feeding in the apparatus used by us was complicated by the fact that since blood serum or cells alone are toxic for bedbugs it was found essential to haemolyse the blood and so prevent separation of its constituent parts. The effect of haemolysis of the blood on development and survival of bedbugs is not known. These difficulties were apparently not encountered by Nicolle & Lwoff (1944) and Lwoff & Nicolle (1944), who have worked on similar lines with *Triatoma infestans*, which they were able to rear to maturity on horse serum plus a mixture of vitamins.

The possibility of interfering with the metabolism of a blood-sucking insect by altering the character of the blood on which it feeds presents a very fruitful field for investigation, especially from an economic point of view. There seems no reason to suppose, for example, that substances will not be found which on oral administration to animals will so upset the metabolism of ectoparasites as to bring about some measure of control.

In any investigation of the nutritional requirements of arthropods which take only sterile blood during the whole course of their life, the fact must not be lost sight of, that they all—as far as they have been investigated—possess intracellular symbionts. These symbionts have no existence outside the arthropod in which they occur, nor do any of them occur in nature outside the host. In addition, they are passed on hereditarily from mother to offspring through the reproductive system, and a complicated mechanism has been developed for this purpose. It has therefore been supposed that the occurrence of the symbionts in these animals is not simply a fortuitous association but that they play a more vital part in the life of the hosts. Their universal occurrence in insects and ticks which live on sterile blood only has given rise to the suggestion that they perform some nutritive function (Wigglesworth, 1939). It is known from the work of Hobson (1933), for example, that sterile larvae of the blow-fly *Lucilia* fail to develop on sterile mammalian blood but do so when sterile yeast extract is added. Further, some evidence exists that sterile blood becomes adequate for such larvae when infected with a symbiont from the blood-sucking hemipteron *Rhodnius prolixus* Stal. (Wigglesworth, 1936). Similarly, Aschner (1932) and Aschner & Ries (1933) have shown that the symbionts in the human body-louse are required for growth and reproduction. Young louse nymphs deprived of their symbionts by ultracentrifugation die in a matter of 6 days, and adult females, arising from final nymphs in which the mycetome has been extirpated, produce no eggs or only a few sterile ones.

Koch (1933) destroyed the symbionts of the beetle *Sitodrepa panicea* by sterilizing the eggs and found that yeast had now to be added to his basic diet, presumably vitamin-free, before satisfactory growth was obtained. Blewett & Fraenkel (1944) extended Koch's work, and though working with unsterile media appear to have shown that the symbionts of *Sitodrepa* and another beetle *Lasioderma* supply vitamins of the B group.

Symbionts, many of them intracellular and as closely associated with their hosts as in blood-sucking hemimetabolous insects, also occur in many other insects which take unsterile food, at least during some part of their life cycle. The role of symbionts in these insects is, on the whole, less clear. In some of them the symbionts appear to be essential and apparently provide accessory food substances. Other hosts appear to develop quite normally when deprived of their symbionts, while others again have mycetomes which contain no symbionts (Glaser, 1930; Wigglesworth, 1939; Steinhaus, 1940).

There is, of course, no reason at all why the same functions should be ascribed to all symbionts. Symbionts were presumably originally true parasites of insects which through evolution became more and more adapted to their hosts and their hosts to them until in some cases a state of true symbiosis was reached. During these ages every conceivable point in the evolutionary chain from parasite to symbiont will have been reached, in some to remain unchanged to the present day, in others to progress and yet in others to regress and even to disappear. Insects themselves have also not remained static through the ages. Some have changed their feeding habits and mode of life, possibly to the detriment of their symbionts. Others have developed highly specialized tastes, e.g. blood-sucking insects. The functions of symbionts are therefore probably as varied as the life and economy of insects themselves.

In the two arthropods with which we have experimented, hereditarily transmitted intracellular symbionts have been demonstrated. In the bedbug, Buchner (1923) showed that they occur in the fat body and in special mycetocytes connected with the gonads and are transmitted to the developing oocytes via the nurse cells. Cowdry (1925) failed to find any symbionts in twenty-five *Ornithodorus moubata*, though he succeeded in demonstrating them in a great number of other ticks including *O. megnini* and *O. turicata*. Later, Mudrow (1933) and Jaschke (1933) showed that they occur in the Malpighian tubes and are transmitted hereditarily. The mechanism of transmission is not known with certainty, but Mudrow (1933) has shown that the symbiont-laden portion of the Malpighian tube establishes very close contact with the ovaries and symbionts then presumably migrate into the developing egg.

Thus the symbionts of *O. moubata* and of the bedbug occupy totally different positions in their respective hosts. It was therefore reasonable to expect that their functions might not be identical. Two avenues of approach to this problem were explored. In the first place an attempt was made to eliminate the symbionts of the bedbug with penicillin, as Brues & Dunn (1945) had succeeded in doing in the cockroach. Secondly, both bedbugs and ticks were fed on animals in whom dietary deficiencies had been induced; the growth and survival rates of the arthropods were measured and their fertility assessed.

Previous work on the bedbug has been mainly concerned with its ecology and has been ably extended and summarized by Johnson (1941), while in *O. moubata* little more has been attempted than the determination of life cycles and the length of life under experimental conditions.

It has been shown by Johnson (1937) that the biology of the bedbug varies on different hosts and indeed on different individual hosts of the same species. From his experiments he concluded that the variation in the results obtained with different hosts may not necessarily be due to the blood itself but possibly to the feeding behaviour of the bugs under experimental conditions. In his experiments, as in ours, bugs were only given one full feed between each moult, whereas in nature a bug may take repeated small meals which may have a different effect. By feeding bugs frequently Janisch (1933), for example, reared the great majority of bugs to the adult stage on a guinea-pig, whereas in our experiment with this animal only 9% of the original first-stage larvae reached maturity. In nature, therefore, one host may be as good as another, contrary to the conclusions drawn under experimental conditions in the laboratory.

We have not been concerned with what may prove to be a good or a bad host in nature but rather with the reaction of the bedbug to certain types of blood consumed under laboratory conditions. We do not claim that our findings are applicable under natural conditions. Since we were primarily interested in the effect of a full blood meal on the bug and not on the suitability of the donor as a host in nature, every effort was made to get as many bugs as possible to feed to repletion.

In our earlier experiments the bugs were weighed before and after meals. It was observed that a larger meal was taken of those bloods on which the bugs grew faster; in consequence the bugs weighed more and more eggs were laid, an observation which is in agreement with that of Johnson (1937). For various reasons we did not continue weighing the bugs, being content to interpret our results in terms of speed of development, survival to the adult stage and mean number of eggs laid per female.

MATERIAL AND METHODS

Cimex lectularius L. A stock of bugs was obtained from Dr G. Booker. Initially the technique described by Johnson (1941) was employed for breeding, but for reasons which could not be determined little success attended these efforts. Following this failure, stocks were maintained satisfactorily by direct feeding on a rabbit's ear. About twenty adult bugs were confined in a tube (2·5 × 1·5 in.), the open end of which was covered with voile. A few pieces of paper, preferably coloured in order to render the eggs easily visible, were placed in the tube. When the bugs were not being fed, stocks were kept at 28° C. in an incubator, the humidity being kept approximately constant by using an open dish of water in the incubator.

Ornithodorus moubata Murray. A number of ticks of South African origin but of mixed strains were obtained from Dr D. Ordman. They were reared and maintained in the same way and under the same conditions as *Cimex*.

Experimental feeding of Cimex *nymphs and adults*

(*a*) *On animals*. The technique described above for feeding bugs for breeding purposes was used in experiments to determine the speed of development on the rabbit. In the case of other animals a portion of skin was shaved or the hair removed with a depilatory, and after thorough washing and drying the tubes with bugs were applied.

(*b*) *Through a membrane*. The membrane consisted of a mouse skin which was prepared as follows: A white mouse was killed with ether and all its hair removed with a depilatory. The animal was then skinned, as much fat as possible scraped off the skin, which was washed in running water for 1 hr. It was finally dried on blotting-paper.

The feeding tube consisted of a glass U-tube of which one arm, of narrow bore, was considerably longer than the other. The shorter arm was widened to form a cup across which the mouse skin was stretched and fixed in position by means of an elastic band. The apparatus was filled by introducing the test medium through the narrow open end and rocking the tube to remove all air bubbles. The long arm was filled almost to the top so that the fluid was pressed against the membrane, distending it slightly.

The feeding tube when full was placed in a constant-temperature water-bath at about 40° C. in such a way that the membrane was just above the water-level. In order to confine the bugs to the membrane a glass tube of slightly smaller bore and open at both ends was placed on it and held in position by a clamp. Early-instar bugs coming into contact with the warm skin usually fed almost immediately. Bugs in later stages of development took longer to feed, and adults had usually to be left for 0·5 hr. or longer before they were replete.

(*c*) *The prefeeding period.* Each nymph and adult bug could not be fed immediately after moulting, since in every group moulting extended over a period of several days. Accordingly, a standard procedure was adopted, namely, to feed the whole group simultaneously 1–2 days after the last moult had occurred.

(*d*) *Pairing of adult* Cimex. The adult bugs were paired by placing one female and one male in a tube containing a piece of dark-coloured paper to which the eggs laid could be attached and be readily visible.

The preparation of media for Cimex

(*a*) *Defibrinated haemolysed blood.* The blood was defibrinated by shaking vigorously, immediately after collection, with glass beads; the clots were removed by straining through gauze. Haemolysis was produced by alternate freezing with dry ice and thawing, the process being repeated once or twice. If it was desired to keep the blood thus prepared in order to evaluate its nutritive value after storage, it was finally frozen solid and stored in this condition in a refrigerator at -5° C.

(*b*) *Heparinized haemolysed blood.* From one to two Toronto units of heparin were added for each ml. of blood, in order to prevent clotting. Such blood was haemolysed in the manner described above.

(*c*) *Serum.* Freshly drawn whole blood was defibrinated and the serum separated after centrifuging. The serum was always used on the same day as it was collected. Care was taken to use only serum which was free from haemoglobin.

(*d*) *Red cells.* Whole defibrinated fresh blood was centrifuged and the red cells collected. The cells were washed several times with normal saline until judged to be free from serum.

(*e*) *Reconstituted whole blood.* Fresh whole blood was defibrinated, the fibrinogen separated, washed with water, dissolved in dilute sodium hydroxide by warming, and after cooling reintroduced into the blood which had in the meantime been haemolysed. The *p*H was adjusted to 7·5 with hydrochloric acid.

(*f*) *Haemolysed red cells in nutrient medium.* Washed cells were haemolysed in a liquid containing a 'nutrient medium' composed of 1% glucose and sodium caseinate; 0·15% salt mixture (Tatum); 10 mg./l. thiamin, riboflavin, nicotinic acid, calcium pantothenate, pyridoxin hydrochloride; 0·33 mg./l. biotin; 25 mg./l. inositol; 25 mg./l. *p*-aminobenzoic acid; 5 g./l. Bacto yeast extract.

(*g*) *Stirred human blood.* Freshly drawn human blood was defibrinated and introduced into the apparatus. The right-hand limb was closed by a rubber cap through which passed a syringe needle reaching to the lower end of the tube. By means of a syringe attached to the needle the blood was pumped in and out, thus stirring the contents of the U-tube and preventing the separation of the cells from the plasma.

(*h*) *Penicillin in blood.* The purified sodium salt of penicillin was taken up in normal saline, and 1 ml. of this saline solution added to 24 ml. freshly prepared defibrinated haemolysed blood. By appropriate dilution of the saline solution concentrations ranging from 40 to 4000 Oxford units/ml. of diluted blood were obtained. The relative proportions of saline and blood were kept constant.

Experimental feeding of Ornithodorus moubata

A portion of the back of a rat was freed from hair by treatment with a depilatory and, after thorough washing, the animal was kept for at least 48 hr. The rat's head was placed in a cloth bag which was secured round its neck so that the ticks could not be disturbed. Ticks were then placed on the cleaned portion of the back. Although the rat made no attempt to dislodge the ticks by scratching, many of the ticks failed to attach themselves to the animal.

Subsequently the following procedure was tried and proved successful: The rat was wrapped tightly in a cloth which was then tied round with string. The cloth was open at the head and tail ends and the ticks were introduced at the open tail end on to the cleaned portion of the skin. They were left on the animal until fully fed, when they dropped off of their own accord.

Cimex lectularius *nymphs*

In all experiments the bugs were examined either once or twice each day and the number which had moulted was recorded. This was most easily done by counting the number of cast pelts. Bugs which did not feed, or did not feed fully, were removed and added to the breeding stock. Bugs which appeared to be fully fed to the naked eye were counted as such. No doubt this assumption constituted a source of error and probably accounted for individual variations in the time taken to moult following a meal.

In certain experiments the weights of bugs were determined and taken into account in assessing the results.

Fertility of adults

The adults of *Cimex* were fed on the same medium or animal which had been used for feeding the nymphs. The number of eggs laid by each female was noted.

Injection of rabbits for experiments with Cimex

(*a*) *With heparin.* Five or ten minutes before the bugs were fed 0·2 ml. of heparin in 0·8 ml. normal saline solution was injected intravenously into the rabbit. This amount is equivalent to a human adult dose of approximately 5000 Toronto units. The clotting time of the rabbit was not determined.

(*b*) *With dicoumarol.* A dicoumarol solution for injection was prepared as described by Overman, Stahmann, Sullivan, Huebner, Campbell & Link (1942). Two or three days before the bugs were to be fed, rabbits were given an intravenous injection of this solution equivalent to 5–10 mg. of dicoumarol. The larger amount was used in the early stages of the experiment, but as both rabbits receiving this dose died it was reduced to 5 mg. This quantity was sufficient to give a prothrombin index (Quick) of about 33% or less 2–7 days following administration.

(*c*) *With penicillin.* Purified sodium salt of penicillin (Schenley) was dissolved in normal saline solution and administered intravenously to a rabbit weighing 2·4 kg. The amount given was sufficient to attain a concentration of 400 Oxford units/ml. of rabbit's blood. The bugs were fed on the rabbit within a few minutes of the administration of penicillin. Blood withdrawn from the rabbit up to 4·5 hr. later still gave evidence of penicillin activity. No further tests were carried out.

Injection of Cimex *with penicillin*

The penicillin was injected into the haemocoele of the bugs, care being taken to inject approximately the same amount of liquid into each bug; although the exact volume used was not determined, it was sufficient to cause the bug to swell up visibly. A large number of fifth-stage stock nymphs were divided into two groups, one being injected with normal saline as a control group and the other being given a solution of penicillin (Schenley), in normal saline of potency 20,000 Oxford units/ml.

Test for penicillin

Qualitative tests for penicillin activity were carried out on agar plates using *Staphylococcus aureus* as the organism.

Induction of deficiencies in rats for experiments with Cimex

(*a*) *Thiamin deficiency*. The diet devised by Kline, Hall & Morgan (1941) had previously been shown by Golberg & Thorp (1943) to be a highly satisfactory method of inducing a pure thiamin deficiency in young rats. Accordingly, two groups of rats were selected and one placed on the deficient diet while the other was placed on the same diet supplemented with thiamin (at a level of 100 μg. of thiamin chloride per 100 g. of diet).

As will be seen in the accompanying growth curves (Fig. 1) the development of deficiency as judged by cessation of growth was quite clear-cut.

(*b*) *Unpurified diets* (*with and without liver extract*) *supplemented by thiamin and succinylsulphathiazole*. The diet of Kline *et al.* includes 1% of sulphite-treated liver extract. Two diets were made up—one with and the other without the liver extract. To each was added thiamin chloride (100 μg./100 g. of diet) and succinylsulphathiazole (2%).

On both diets there was impairment of growth, as can be seen clearly from the growth curves given in Fig. 1. However, blood counts failed to reveal any leucopenia or granulocytopenia.

(*c*) *Purified diet containing succinylsulphathiazole*. The diet S–1 of Welch & Wright (1943) was used as a model in devising a purified diet for use with succinylsulphathiazole (2%). The ingredients of the diet were: sucrose (74%), casein (B.D.H. fat and vitamin-free) (18%), arachis oil (2%), salt mixture (Hubbell, Mendel & Wakeman, 1937) (4%), choline chloride (0·2%), vitamins A, D, E, B_1, B_2, B_6, nicotinic acid and calcium pantothenate as given by the above authors.

In this instance, too, there was marked impairment of growth. Furthermore, blood counts carried out on the thirty-sixth and fifty-eighth days revealed a marked leucopenia and granulocytopenia in all the animals of this group.

Estimation of thiamin in rat blood

The method of preparing a protein-free extract of rat blood was adapted from Friedemann & Kmieciak (1943).

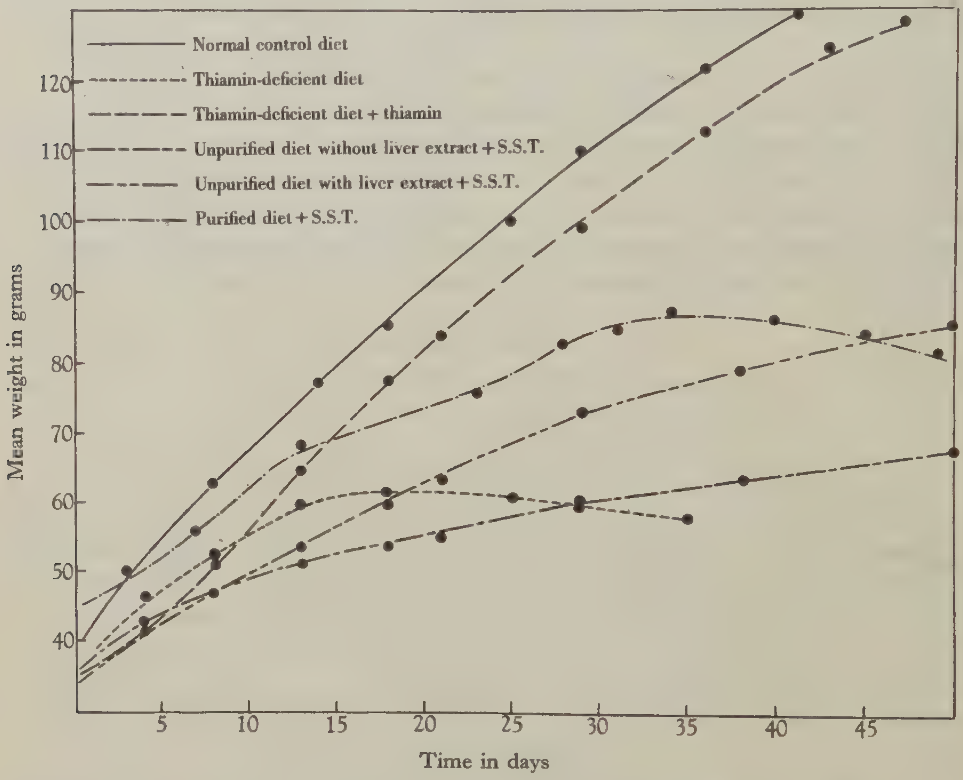

Fig. 1. Weight curves of rats on experimental diets.

EXPERIMENTAL

A. *Development of bedbugs when fed directly on various animals*

From Table 1 it will be seen that under the experimental conditions some types of blood are more suitable for the development and survival of bedbugs than others. The hosts may be roughly divided into two similar groups, namely: (1) guinea-pig and horse, and (2) rabbit, rat and man. Bugs fed on group 1 developed more slowly and less of them reached maturity than those fed on group 2. There is not much to choose between individuals in each group, though development was slightly faster on man and rabbit than on rat. The largest percentage of bugs reached maturity on man. In the three animals of group 2 the mean number of eggs per female, laid after one feed, was approximately the same.

Table 1. *Experiments with bedbugs fed directly on various animals*

P = percentage of fully fed nymphs which moulted from the previous stage.
T ≐ mean and standard deviation of the time (in days) taken to moult after feeding.

Animal	Stages of development											Original 1st nymphs reaching adult stage %	No. of pairs	Mean and S.D. of no. of eggs per female after one feed	Remarks
	I	II		III		IV		V		Adult					
	No. fully fed	P	T	P	T	P	T	P	T	P	T				
*Guinea-pig	92	74	4·1 ± 0·35	75	4·6 ± 0·80	85	5·1 ± 0·64	84	5·1 ± 0·87	50	6·6 ± 0·95	10	—	—	—
*Horse	48	98	3·7 ± 0·47	97	4·5 ± 0·44	100	5·7 ± 1·0	100	4·9 ± 0·41	100	6·5 ± 0·38	29	—	—	Adults refused to feed
Rabbit	65	89	3·5 ± 0·63	93	3·8 ± 0·42	97	4·0 ± 0·54	100	4·1 ± 0·56	100	5·4 ± 0·71	43	11	14·3 ± 4·0	—
*Rat	96	95	4·1 ± 0·40	100	4·4 ± 0·98	96	4·1 ± 0·40	97	4·5 ± 0·55	96	6·1 ± 0·73	67	21	10·4 ± 2·5	—
*Man	43	100	3·9 ± 0·37	100	3·8 ± 0·24	100	3·7 ± 0·45	100	4·2 ± 0·39	97	5·5 ± 0·45	74	13	11·5 ± 1·7	—

* Sum of two experiments.

Table 2. *Experiments with bedbugs fed through a membrane on freshly drawn, defibrinated, haemolysed blood of various animals*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = mean and standard deviation of the time (in days) taken to moult after feeding.

Source of blood	Stages of development											Original first nymphs reaching adult stage %	No. of pairs	Mean and S.D. of no. of eggs per female after one feed	Remarks
	I	II		III		IV		V		Adult					
	No. fully fed	P	T	P	T	P	T	P	T	P	T				
Man	37	95	3·9 ± 0·21	97	4·2 ± 0·57	100	4·5 ± 0·33	96	4·8 ± 0·61	88	6·1 ± 0·45	38	—	—	Adult bugs refused to feed
Rabbit	43	89	3·6 ± 0·24	93	3·7 ± 0·32	58	4·4 ± 0·57	100	4·8 ± 0·47	80	7·3 ± 0·44	9	2	—	Adults died without laying eggs
Horse	37	100	3·7 ± 0·25	76	4·9 ± 0·60	57	5·7 ± 0·35	25	6·5 ± 0·0	0	—	—	—	—	All bugs dead by time Vth stage was reached

Table 3. *Experiments with bedbugs fed through a membrane on stored, defibrinated, haemolysed blood of various animals*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = mean and standard deviation of the time (in days) taken to moult after feeding.

Source of blood and length of storage at −5° C.	Stages of development											Original first nymphs reaching adult stage %	No. of pairs	•Mean and S.D. of no. of eggs per female after one feed	Remarks
	I	II		III		IV		V		Adult					
	No. fully fed	P	T	P	T	P	T	P	T	P	T				
Man: Fresh for I, 8 days for II, 17 days for III, 24 days for IV, 33 days for V, and 44 days for adults	44	100	3·9 ± 0·23	98	4·3 ± 0·47	89	4·8 ± 0·48	64	5·8 ± 0·78	94	6·4 ± 0·59	34	2	4	—
Man: 13 days for I, 20 days for II, 27 days for III, 37 days for IV, and 51 days for V	39	89	4·8 ± 0·78	96	4·8 ± 0·40	82	5·1 ± 0·98	80	5·4 ± 0·81	100	7·2 ± 0·42	2	—	—	Adults refused to feed
Rabbit: Fresh for I, 7 days for II, and 16 days for III	35	88	3·6 ± 0·41	50	3·8 ± 0·18	0	—	—	—	—	—	—	—	—	All bugs dead by time IIIrd stage was reached
Rabbit: 16 days for I, 21 days for II, and 28 days for III	26	96	3·8 ± 0·43	96	4·2 ± 0·44	67	5·0 ± 0·0	0	—	—	—	—	—	—	All dead by time IVth stage was reached
Horse: Fresh for I, 7 days for II, 14 days for III, 24 days for IV, and 36 days for V	108	93	3·75 ± 0·65	66	5·4 ± 0·67	42	7·3 ± 0·46	21	7·7 ± 1·10	33	10·0	1	—	—	All dead by time adult stage was reached
Bugs which failed to moult to III on stored horse blood fed on a rabbit	No. of bugs			23	4·0 ± 0·0	91	3·8 ± 0·30	94	4·3 ± 0·36	88	5·5 ± 0·52	65	5	13·2 ± 1·2	—
Bugs which failed to moult to IV on stored horse blood fed on a rabbit	No. of bugs					17	4·5 ± 0·44	100	4·1 ± 0·58	100	5·5 ± 0·50	76	—	—	—

B. *Effects of feeding bedbugs through a membrane on the blood of various animals*

In comparison with direct feeding, the bedbugs did not respond well when fed through a membrane on freshly drawn, defibrinated, haemolysed blood (Table 2). The speed of development was hardly affected except for the time taken to moult from fifth nymph to adult. The mortality, however, was greatly increased. This points to the presence of toxic substances in the blood, possibly released in the course of its preparation for membrane feeding. As in direct feeding, the bugs fared worse on horse blood than on human or rabbit blood. The adults from the human and rabbit blood groups either failed to feed after repeated attempts or else died without laying any eggs.

C. *Effects of storage of blood on bedbugs fed through a membrane*

Table 3 shows the effect of short and long storage (at approximately −5° C.) of blood of various animals on the development of the bedbug. Storage of human blood increases its toxicity and decreases the rate of development significantly as compared with direct feeding, except for the time taken to moult to the second and third stages. Longer storage tends to exaggerate these differences, especially as far as mortality is concerned. We succeeded in feeding two pairs of adults on stored blood, and in each case four eggs were laid. It is possible that reproduction is also affected. Rabbit blood on storage proved to be highly toxic and no bugs survived to the fourth stage; up to this point the rate of development was not appreciably affected. Horse blood on storage surprisingly enough did not increase in toxicity, but when compared with direct feeding the rate of development was decreased. Second- and third-stage bugs which failed to moult on stored horse blood were fed on a rabbit. They immediately recovered and presented a rate of development which, taken together with the lower percentage mortality for the rest of their cycle, was equal to that of bugs fed directly on a rabbit. Moreover, their reproductive capacity was not impaired.

D. *Effects of modified blood on bedbugs fed through a membrane*

In Table 4 are shown some results of feeding modified blood through a membrane to bedbugs. Many other experiments were carried out on the same lines before it was discovered that the addition of as little as 4% of normal saline to blood fed through a membrane resulted in the death of all the bugs by the time the third stage was reached. In an attempt to ascertain whether defibrination affected the nutritive value of blood, an attempt was made to restore the fibrin to the blood by dissolving it and adding to the original volume of blood. The resulting blood again proved highly toxic. It seemed possible that haemolysis of the red cells might possibly liberate toxic substances and so account for the high mortality (see Table 2). An experiment with defibrinated blood was therefore carried out in which the blood was agitated to ensure mixing of cells and serum while the bugs were being fed. However, all the bugs died by the time the third stage was reached. It is possible that the stirring was not effective and the bugs imbibed too much serum or too many cells.

Blood was withdrawn from the rabbit which had received penicillin intravenously a short while before; when fed to bugs it also proved highly toxic. The cause of this toxicity is unknown. That it was not due to a specific effect of the penicillin is evident from experiments reported in Table 6, in which it was shown that *direct* feeding on a penicillin-treated rabbit had no such effect. One hour after feeding the bugs some were crushed and the expressed liquid tested for penicillin activity. None was found, although the rabbit's blood still showed activity.

E. *Effects of blood fractions and supplements on bedbugs fed through a membrane*

In Table 5 are summarized the results of feeding bugs on fractions of blood and on the same fractions plus supplements. With the exception of horse serum plus 5% papaic liver digest, and of horse cells plus nutrient medium the bugs fed readily enough, but in all cases the media proved highly toxic. Bugs given horse cells plus saline did not moult and were fed directly on rabbit, thus completing their life cycle in a normal manner.

F. *Attempts to modify the blood of the host by injection of various substances*

Attempts to interfere with the bedbugs' metabolism by injection of various substances into a rabbit are recorded in Table 6. On the whole very little difference can be seen between the three experiments except in the times taken to moult in the final stages. The bugs fed on the rabbit injected with dicoumarol were unfortunately not tested for egg-laying. When comparing bugs fed on a normal rabbit with those fed on the dicoumarol-treated rabbit, a significant difference can be shown to exist between the times taken to change into fourth and fifth nymphs. Reproduction, where it was tested, did not appear to be greatly affected. The mortality in all experiments, but especially in the bugs fed on the rabbit injected with heparin, was higher than normal. When the great disturbance brought about by the dicoumarol in the rabbit is considered (Table 7), it is surprising that the development of the bugs was so little affected.

The bugs fed on the penicillin-injected rabbit appeared to be affected least of all. Preparations made from bugs which had been reared in this way showed that the symbionts were apparently unaffected.

G. *Attempts to destroy the symbionts by injection of bedbugs with penicillin*

The penicillin was injected into the haemocoele of the bugs. Of a large number of fifth-stage stock nymphs some were injected with normal saline to act as a control and others with penicillin (Schenley) so diluted with normal saline as to give a concentration of 20,000 Oxford units/ml. Approximately the same amount of liquid was injected into each bug. The exact amount is not known but was sufficient to cause each bug to swell up visibly. Of the survivors, twelve which had received saline only and fifteen which had penicillin were fed to repletion on a thiamin-deficient rat. The former took 6·0 ± 0·47 days to moult to the adult stage and the latter 6·9 ± 0·47 days. On a normal rat fifth-stage nymphs take 6·1 ± 0·73 days, so it would appear that a single injection of penicillin in the fifth stage does not appreciably affect the time taken from feed to moult. Injection with penicillin of each nymphal stage from one to five might possibly reduce the symbionts but we did not attempt this. Finally, bugs which had received an injection of penicillin and which had moulted to the adult stage were dissected, and apparently normal symbionts were observed in the ovaries and fat body, though we could not say for certain if they were reduced in number.

Table 4. *Experiments with bedbugs fed through a membrane on freshly drawn blood modified in certain respects*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = Mean and standard deviation of the time (in days) taken to moult after feeding.

Source of blood and modifications	Stages of development						Remarks
	I	II		III		IV	
	No. fully fed	P	T	P	T	P	
Rabbit: injected with penicillin, blood then withdrawn, defibrinated, and haemolysed for each feed	52	69	4·05 ± 0·12	50	5·1 ± 0·72	0	All dead before IVth stage was reached
Rabbit: blood freshly drawn, defibrinated, haemolysed, 4% normal saline added for each feed	48	75	4·6 ± 0·49	3	5·0	0	All dead before IVth stage was reached
Man: blood freshly drawn, defibrinated, haemolysed and original fibrin added in solution	32	94	4·4 ± 0·47	87	4·8 ± 0·59	0	All dead before IVth stage was reached
Man: blood freshly drawn, defibrinated only and stirred while bugs were feeding	41	98	4·4 ± 0·45	73	5·0 ± 0·50	0	All dead before IVth stage was reached

H. *Attempts to modify the blood for bedbugs by dietary changes in the experimental host* (*rat*)

(1) *Folic acid deficiency*

In the first two experiments rats were placed on an unpurified diet containing succinylsulphathiazole, with and without added liver extract. Fifty-five days later and a day before we commenced to feed the bugs an attempt was made to collect blood from the animals both by heart puncture and from the tail. In both experiments the blood showed a tendency to clot immediately it was withdrawn, and it was not possible to make accurate blood counts. Nevertheless, it was possible to ascertain that no leucopenia or granulocytopenia existed. The bugs fed on two of these rats developed at the normal rate but laid less eggs per female (Table 8).

In the next experiment rats were placed on a purified diet containing the same concentration of succinylsulphathiazole as before (2%). The result here was substantially the same as in the first two experiments, namely, a reduction in the

Table 5. *Experiments with bedbugs fed through a membrane on various fractions of blood and supplements to fractions*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = mean and standard deviation of the time (in days) taken to moult after feeding.

Source of blood, fractions and supplements	Stages of development											Original first nymphs reaching adult stage %	Remarks
	I	II		III		IV		V		Adult			
	No. fully fed	P	T	P	T	P	T	P	T	P	T		
*Man, fresh serum only	81	44	5·9 ± 1·0	0	—	—	—	—	—	—	—	—	81 out of 100 bugs fed, none survived beyond the IInd stage
*Man, fresh, washed, undiluted, haemolysed red cells	68	44	4·3 ± 0·38	0	—	—	—	—	—	—	—	—	All dead before IIIrd stage was reached
Horse, fresh serum + 5 % papaic liver digest	8	25	5·0	0	—	—	—	—	—	—	—	—	8 out of 50 bugs fed; all dead before IIIrd stage was reached
Horse, fresh washed red cells, defibrinated and haemolysed + 5 % papaic liver digest	40	82	4·3 ± 0·44	0	—	—	—	—	—	—	—	—	40 out of 50 bugs fed; all dead before IIIrd stage was reached
Horse, fresh washed red cells, defibrinated, haemolysed + normal saline replacing serum	61	41	4·5 ± 0·55	0	—	—	—	—	—	—	—	—	61 out of 75 bugs fed; all dead by time IIIrd stage was reached
Bugs which fed in Ist stage on red cells + saline but did not moult, fed on rabbit	12	75	5·3 ± 0·47	75	4·8 ± 0·62	100	4·2 ± 0·24	100	4·5 ± 0·0	100	5·2 ± 0·62	25	—
Horse, fresh washed red cells, defibrinated, haemolysed + nutrient medium†	14	57	4·4 ± 0·47	0	—	—	—	—	—	—	—	—	14 out of 50 bugs fed; all dead by the time IIIrd stage was reached

* Sum of two experiments. † For composition of nutrient medium see 'Methods'.

Table 6. *Experiments with bedbugs fed directly on a rabbit injected with various substances*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = mean and standard deviation of the time (in days) taken to moult after feeding.

Substances injected intravenously	Stages of development											Original first nymphs reaching adult stage %	No. of pairs	Mean and S.D. of no. of eggs per female after one feed
	I	II		III		IV		V		Adult				
	No. fully fed	P	T	P	T	P	T	P	T	P	T			
Heparin, 0·2 ml., 15 min. before each feed	30	90	3·5 ± 0·17	100	4·7 ± 0·45	100	4·1 ± 0·75	83	5·1 ± 0·82	100	7·3 ± 1·70	10	1	12
*Dicoumarol, 10 mg. for I and II feeds and then 5 mg. on three occasions during course of experiment	87	70	4·2 ± 0·67	89	4·7 ± 1·07	85	5·8 ± 1·20	82	5·2 ± 0·35	80	6·4 ± 1·10	28	—	—
Penicillin, to give a concentration of 400 Oxford units in rabbit's blood, shortly before each feed	59	88	4·6 ± 0·81	96	4·4 ± 0·57	98	4·5 ± 0·96	89	4·2 ± 0·66	95	4·9 ± 0·70	32	6	9·0 ± 1·3

* See Table 7 for further details.

number of eggs laid and no apparent effect on the rate of development. In this experiment blood was collected successfully on two occasions. Sixteen days after the rats had been placed on the purified diet plus succinylsulphathiazole the feeding of the bugs was started, and when they had reached the fourth stage the first blood count was made. This revealed that the white cells of the rats had undergone reduction to 5800/cu.mm. in one case and 3200/cu.mm. in the other. Normal stock rats from which our experimental animals were taken show a leucocyte count of over 10,000/cu.mm. The second blood count was made at the conclusion of the bug-feeding experiment, and by that time the white cell count of the rats was reduced to 250/cu.mm. The growth curves of the deficient animals, taken in conjunction with the marked effect of succinylsulphathiazole on the blood picture, again illustrated how difficult it is to influence the development of the bedbug by bringing about changes in the host's blood *in vivo*.

Table 7. *Details relating to the blood changes brought about in rabbits injected with dicoumarol and on which bugs (Table 6) were fed*

Rabbit no.	Date of injection of dicoumarol and dose	Date of feeding of bugs and stage in which they were fed	Date of estimation of prothrombin index and value of index	Remarks
1 and 2	30. viii. 45 10 mg.	1. ix. 45 I	6. ix. 45 5 %	—
1 and 2	5. ix. 45 10 mg.	7. ix. 45 II	—	Rabbits 1 and 2 died before third-stage bugs could be fed
3	14. ix. 45 5 mg.	17. ix. 45 III	17. ix. 45 33 %	—
3	25. ix. 45 5 mg.	27. ix. 45 IV	—	—
3	9. x. 45 5 mg.	11. x. 45 V	11. x. 45 30 %	—

(2) *Thiamin deficiency*

The results with thiamin-deficient rats reveal that the rate of development of the bedbugs was not affected but that egg-laying was rather drastically reduced (Table 8). Moreover, it was noticed that eight out of the eleven eggs produced in the second experiment were small and shrunken and corresponded to what German writers have described as 'taub' eggs (Johnson, 1941). One of the normal eggs subsequently proved to be fertile and an apparently normal nymph hatched out; the other two normal eggs and all the 'taub' eggs proved to be sterile. Seven pairs of the bugs reared on thiamin-deficient rats were then fed once on a normal rat and each laid an average of five eggs. Only two of the eggs were of the 'taub' variety and 55 % hatched. Thus, by returning the bugs to a normal rat, the number of eggs laid per female was increased and the proportion of sterile eggs and of 'taub' eggs considerably reduced.

Table 8. *Experiments with bedbugs fed directly on rats subjected to various deficient diets*

P = percentage of fully fed nymphs which moulted from the previous stage.
T = mean and standard deviation of the time (in days) taken to moult after feeding.

Rat diet	Stages of development											Original first nymphs reaching adult stage %	No. of pairs	Mean and S.D. of no. of eggs per female after one feed
	I	II		III		IV		V		Adult				
	No. fully fed	P	T	P	T	P	T	P	T	P	T			
Unpurified + casein + liver + succinylsulphathiazole	45	100	4·35 ± 0·48	82	4·7 ± 0·75	78	4·1 ± 0·26	100	4·75 ± 0·92	90	5·6 ± 0·75	42	6	5·2 ± 1·9
Unpurified + casein + succinylsulphathiazole	60	83	4·5 ± 0·66	73	4·4 ± 0·86	92	4·1 ± 0·29	100	4·6 ± 0·82	86	5·9 ± 0·39	42	5	6·2 ± 2·1
Purified + succinylsulphathiazole	65	95	4·1 ± 0·30	76	4·5 ± 0·72	93	4·1 ± 0·58	96	4·5 ± 0·25	68	6·0 ± 0·0	23	5	6·0 ± 3·3
Thiamin deficient	45	98	4·2 ± 0·46	85	4·2 ± 0·44	100	4·5 ± 0·92	86	5·1 ± 0·62	58	8·0 ± 0·89	33	7	1·3 ± 0·45
Thiamin deficient (repeat)	62	98	4·5 ± 0·50	96	4·3 ± 0·54	97	4·3 ± 0·68	94	4·7 ± 0·77	68	6·0 ± 1·10	34	8	1·4 ± 1·0

J. *Attempt to modify the blood for* Ornithodorus moubata *by dietary changes in experimental host (rat)*

First-instar *O. moubata* nymphs of unknown age were divided into one lot of twenty which was fed on a normal rat and one lot of twenty-four fed on a thiamin-deficient rat. Up to date the experiment has been carried through to the third nymphal stage. The results are summarized in Table 9.

Table 9. *Development of* Ornithodorus moubata *when fed on normal and thiamin-deficient rats*

Host	Stages of development					
	I	II		III		
	No. of fully fed Ist stage nymphs	Mean days taken to moult to IInd stage and S.D.	% which moulted	Mean days taken to moult to IIIrd stage and S.D.	% which moulted	Mean weight of tick 7 days after feeding in IIIrd stage mg.
Normal rat	20	9·2 ± 0·4	96	8·6 ± 0·7	100	19
Thiamin-deficient rat	24	11·6 ± 1·4	100	14·0 ± 1·2	90	11

It is obvious from Table 9 that in contrast to the bedbug the development of the tick is adversely affected by feeding on a thiamin-deficient animal. Furthermore, there is an obvious visible difference in the size of the ticks in each group, and this is plainly seen in the mean weights. The percentage which moult is approximately the same in the two groups.

It is of interest to record that the bite of the tick had a much more severe reaction on the thiamin-deficient animal than on the normal rat. Both animals showed a reaction, haemorrhage into the skin, immediately after the ticks had dropped off. In the case of the bite of second-stage nymphs the skin lesions persisted for 2 days in the thiamin-deficient rats, whereas they disappeared from the skin of the normal rat in about 24 hr. When third-stage nymphs were fed the difference in the reactions was even more pronounced. In the normal rat only a faint reaction was still visible after 24 hr., whereas in the thiamin-deficient rat the haemorrhage persisted for about 4 days.

DISCUSSION

We have shown that it is possible to rear bugs from the first nymphal instar to the adult by feeding them through a membrane. If the blood is freshly drawn for feeding in each instar their rate of development does not differ very much from that of bugs fed on an animal. The great majority of adult bugs reared by feeding *in vitro*, however, either refused to feed or died before laying any eggs. In addition, the mortality in *in vitro* feeding is considerably higher. It appears from this that defibrinated, haemolysed blood does not lose any of its nutritive properties, but becomes toxic for bedbugs. This is further shown by storing such blood over relatively long periods when the mortality is increased.

Various explanations for the relatively poorer response to *in vitro* feeding suggest themselves: (*a*) Liberation of toxic substances during defibrination and haemolysis. (*b*) The smallest amount of blood which could be used conveniently for membrane feeding was 15 ml. The bugs on piercing the membrane thus came into contact with a much larger volume of blood than when piercing the skin of an animal. In this way the salivary juice of the bug would immediately be greatly diluted in membrane feeding, whereas judging by the skin reaction to bites on living animals the salivary juice remains localized for some time. It is possible that the salivary juice plays some part in conditioning blood before it is imbibed by the bug in nature. (*c*) The possibility must be considered that when feeding on an animal the bedbug may absorb more than blood alone. (*d*) Differences between the capillary blood consumed by the bug in nature and the venous blood fed to it in our experiments might be sufficiently great to account for the effects observed. (*e*) In the preparation of the mouse skin for membrane feeding a depilatory was used, and though the skin was washed very thoroughly before use, some traces of toxic substances may have been left behind; furthermore, the depilatory certainly removes more than hair only from the skin.

It is rather surprising to find that serum or red blood cells alone are so toxic for bedbugs. While detailed observations were not made, it appeared that when fed on these substances an unduly large proportion of bugs were killed by rupture of their intestines. Attempts to replace blood fractions failed. The investigation of the bedbug's nutritional requirements by feeding through a membrane holds great promise, but much more work of a basic character is required before attempts to replace blood can be considered.

The most successful experiments in modifying blood *in vivo* for bedbugs were those in which the hosts were fed on deficient diets. In all these experiments, although the rate of development was not notably influenced, egg production was affected, especially in the case of bugs reared on thiamin-deficient rats. In addition, in one experiment at least, most of the eggs produced were of the 'taub' variety. Cragg (1923) and Mellanby (1939) have shown that such eggs are unfertile—as ours also proved to be—and are produced when the sperm of the male becomes reduced. Cragg (1923) considers that the number of eggs laid by the female is not only dependent on the state of nourishment of the female but also of the male. The reduced egg production and the sterility of the eggs resulting from bugs fed on thiamin-deficient rats might therefore be due to malnutrition in the male only. We hope to obtain information on this point from experiments now under way.

A test carried out on the blood of the rats used during this experiment failed to reveal any trace of thiamin. It is, however, uncertain whether the effect on reproduction is to be ascribed to lack of thiamin in the rat's blood, as it might equally well have been caused by the accumulation of toxic metabolites such as pyruvic acid in the blood of the host. Against the latter view may be set the approximately normal rate of development shown by the bugs reared on the blood of thiamin-deficient rats. This would appear to lend support to the view that the symbionts synthesize vitamins such as thiamin, since it is inconceivable that the bedbug can develop without thiamin. Folic acid is required by at least three insects, namely, the larva of *Aedes aegypti* (Golberg, de Meillon & Lavoipierre, 1944), *Tenebrio molitor* and *Ephestia kuehniella* (Fraenkel & Blewett, 1946). It is probably necessary for the bedbug as well. If this is so then the succinylsulphathiazole experiments would lead one to believe that the symbionts produce folic acid as well.

Experiments with *Ornithodorus moubata* fed on a thiamin-deficient rat have not gone far enough to allow an opinion to be expressed on reproduction, but it is evident that the rate of development is decreased and the ticks are smaller than those reared on a normal animal. This is of interest and lends support to the view that symbionts, even in arthropods which take only sterile blood as food, do not necessarily perform the same functions.

Shannon (1943) reported that thiamin, when taken orally, relieved the itch of mosquito bites and prevented further biting. Wilson, Mathieson & Jachowski (1944) have not been able to confirm this observation. In our experiments, *O. moubata* seemed to feed on both normal and thiamin-deficient rats with equal ease, but the deficient rats showed a severe and persistent skin reaction which was not evident in normal rats. As this reaction was produced in two different deficient rats on two separate occasions, we feel justified in believing that it is the result of thiamin deficiency.

Our experiments with two quite unrelated arthropods show quite plainly that both are adversely affected by feeding on deficient animals; the bedbug in the very vital function of reproduction and *O. moubata* in size and rate of development, at least. It is generally believed that ectoparasites increase beyond the normal degree of infestation on animals or human beings suffering from malnutrition of one sort or another. Typhus, for example, is said to follow in the wake of famine, and it is a matter of common observation that lice may increase to an enormous extent on dogs which are in bad condition. Attempts have recently been made to place such beliefs and observations on a more accurate scientific basis. Searls & Snyder (1939) showed that when vitamin A was withheld from a rat's diet the lice (*Polyplax spinulosa*) increased in numbers far beyond those found on rats receiving a full diet. Gyorgy (1938) came to the conclusion that riboflavin has a more specific effect on immunity from rat lice. This last author took into consideration the question of activity of the host and found that the riboflavin-deficient rats which had acquired large numbers of lice were by no means inactive or weakened and cleaned themselves in a normal manner. On the other hand, rats deficient in pyridoxin ultimately died after a prolonged period of muscular inactivity and yet did not become unduly lousy. Buxton (1939) has given reasons for believing that in human beings, at least, ordinary daily activity, such as combing, washing, laundering and scratching, must be responsible for a large proportion of deaths amongst lice. We feel, therefore, that the importance of activity on the part of the host in reducing the numbers of ectoparasites is not easily dismissed.

SUMMARY

1. *Cimex lectularius* can be raised to maturity from the first-stage nymph by feeding through a membrane on defibrinated, haemolysed blood of man and rabbit. The rate of development is not very different from that of bugs reared by feeding directly on these hosts, but the mortality is greater, and adults generally refuse to feed or die shortly after feeding. In one instance a few eggs were laid after feeding through a membrane.

2. It was found that even small changes in the blood fed through a membrane led to the death of the bugs in the early stages of development. On the other hand, quite extensive changes brought about in blood *in vivo* by the injection of dicoumarol and heparin appeared to have little if any effect on the development of nymphs fed directly on the animal so treated.

3. Serum or blood cells alone fed through a membrane are highly toxic for bedbugs. Serum could not be replaced by papaic liver digest or normal saline, nor could red cells be replaced by 5% papaic liver digest or a nutrient medium.

4. The symbionts of the bedbug are apparently unaffected by feeding on a rabbit injected with penicillin. It was shown that the penicillin was inactivated in the bedbug's intestine within an hour of feeding. No success attended efforts to kill the symbionts by injection of penicillin into the haemocoele of fifth-stage nymphs.

5. Bedbugs fed on thiamin and folic acid-deficient rats develop at approximately the normal rate, the mortality is slightly increased and reproduction markedly affected. In the case of the thiamin-deficient rat most eggs laid are sterile and of the 'taub' variety.

6. The normal rate of development on a thiamin-deficient rat suggests that the symbionts of the bedbug produce thiamin. They also probably produce folic acid.

7. *Ornithodorus moubata*, when fed on a thiamin-deficient rat, are smaller than normal and the rate of development is significantly decreased.

8. The bite of *O. moubata* produces a more pronounced haemorrhagic skin reaction in a thiamin-deficient rat than in a normal one.

9. The economy of two blood-sucking arthropods, *O. moubata* and *Cimex lectularius*, is adversely affected by feeding on deficient animals when the influence of activity of the host is definitely excluded.

We are indebted to Miss L. O'C. Black for technical assistance; without her patience and reliability much of the work could not have been completed. We wish to thank Dr G. Booker for supplies of *Cimex lectularius* and Dr D. Ordman for stocks of *Ornithodorus moubata*. Lastly, we gratefully acknowledge the help and advice given by numbers of our colleagues on the staff of this Institute.

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VISUAL RESPONSES OF MOSQUITOES ARTIFICIALLY RENDERED FLIGHTLESS

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(With Six Text-figures)

INTRODUCTION

The work of Kennedy (1940) showed that flying mosquitoes (*Aëdes aegypti*) orientate themselves inaccurately but consistently away from the source of a horizontal beam of artificial light and also that when presented with several moving black stripes they tend to face one or other of them. This characteristic *negative* phototaxis of mosquitoes during daytime has either been observed or suggested by numerous previous workers, though not with the same experimental clarity. It is also known that mosquitoes of many species, if not all, tend to leave daytime shelters at dusk and move towards comparatively brightly illuminated exits. There is thus an apparent *positive* phototaxis in the failing light of dusk. The studies reported in this paper were made in an endeavour to determine more exactly the mechanism of orientation of mosquitoes to light and darkness and to observe experimentally if there was a change of direction of taxis at dusk. A large series of observations made with flying mosquitoes by quite different methods will be reported in another paper.

The basic method in all experiments reported here was to render the mosquito flightless so that its movement towards light or darkness could be easily followed, and its track recorded. The fact that an insect which normally flies is forced to respond to stimuli entirely by pedal locomotion may not be exactly natural, but it enables us to analyse behaviour from a new type of data, always keeping in mind the nature of the experimental conditions. How far the behaviour of walking mosquitoes corresponds to or differs from the natural behaviour of flying ones is a matter for further investigation.

MATERIAL AND METHODS

Two species of mosquitoes, *Culex* (*Culex*) *molestus* Forskål and *Anopheles maculipennis atroparvus* van Thiel, were used, cultures of which have been maintained in this laboratory. The specimens of *Culex molestus* were sugar-fed and most of the specimens used had laid the first and only batch of autogenous eggs. Those of *Anopheles maculipennis* were in various gonotrophic stages, unfed, freshly fed on blood, semi-gravid and fully-gravid.

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The mosquitoes were rendered flightless in most of the experiments by removing the wings with clean cuts with needles or fine scissors across the wing expanse at a little distance from the base so that the base was invariably preserved at least up to the region of the humeral cross-vein. In some critical experiments, instead of removing the wings the latter were either stuck together with a minute quantity of shellac placed at their tips or were tied together lightly with a piece of hair. Mosquitoes were lightly etherized to facilitate these operations and experiments were carried out at least an hour later, by which time the animals had completely recovered. Experiments have been carried out both in artificial and in natural light; the details of the experimental conditions are given in individual cases.

No attempt was made to precondition the mosquitoes, but in some of the experiments with artificial light it was noted that specimens which had been kept for an hour or two in darkness seemed to respond more quickly (i.e. appeared to be more sensitive than those kept in daylight); such animals were kept covered with black paper for a short period prior to experimentation. No change in the kind of response resulted from this treatment; subsequent responses were, however, quicker.

EXPERIMENTS

(1) *Observations in a white arena with black vertical bands of different widths*

The apparatus used was as follows: A glass stage, 20 cm. square, was set up on a metal frame and an electric lamp, 60 W., was placed about 16 cm. below it. The top of the glass stage was covered with a sheet of white paper so that when viewed from above it appeared fairly evenly illuminated. A glass cylinder, 7 cm. tall and 7 cm. diameter, lined on the inside with thin white cartridge paper, was inverted over the stage. The whole apparatus was so placed in the darkened laboratory that little light from any other source influenced the behaviour. Vertical strips of black matt paper of varying widths were pasted inside the white wall of the cylinder. The 'wingless' mosquito was then placed on the stage and the cylinder rapidly inverted over it in such a way that the insect was approximately at the centre of the arena. The roof of the cylinder was faintly marked with blue pencil with four equidistant diametrical lines to facilitate placing the cylinder at certain definite angles in relation to the axis of the insect and the position of the black bands. In a typical response the 'shock' of handling the mosquito was sufficient to keep it quiet on the stage for a few seconds so that the manipulation of the cylinder presented no difficulty.

(*a*) 90° *width stripes*. In the first series of experiments a vertical black stripe exactly covered a quarter (i.e. 90°) of the inner surface of the cylinder. The 'wingless' mosquito was put on the stage by tapping it off the container in which it was kept or by dropping it from the tip of a pencil or a finger upon which it was previously picked up. In a typical satisfactory trial, the mosquito would start moving towards the wall after about 10 sec. During this interval the cylinder was inverted over it, centred and so adjusted that one edge of the black stripe subtended a definite angle from the axis of the head towards the left or the right eye. Experiments were carried out at angles of 0 (i.e. directly in front), 45 and 90° to one side. Tests were also carried out with the specimen directly facing the centre of the band or facing exactly in the opposite direction.

In a few seconds the mosquito would begin to crawl and from the very beginning it would move towards the black band. The track followed was immediately recorded on a suitably marked paper. On reaching the base of the wall the mosquito would walk up the wall, very often perpendicularly but sometimes diagonally upwards—at the top of the wall movement usually stopped.

Typical responses of a female *Anopheles maculipennis* are illustrated in Fig. 1. The continuous lines indicate the tracks followed when the right eye was towards the black band and the broken line when the left eye was towards it. When the edge of the dark band was directly in front (A) and when it was 45° to one side (B), the insects in almost all cases moved towards the black band. When the edge was at 90° to one side (C), the response became a little uncertain and at 135° (D), more so. *A. maculipennis* became more uncertain than *Culex molestus* at both these angles but the differences are not very significant. Considering both species together, about 50% of the movement was towards the black band at 90° and about 25% at 135°. When the black band was directly in front (E), there was usually no marked curvature, the specimen walking more or less straight towards the central part of the band.

When the band was directly behind (D, 135°) the specimens walked straight ahead in the majority of cases, and behaved as though they did not detect the band. But in about 25% of cases, as stated above, movement towards the black band did take place. In such cases this response occurred invariably *after* the specimen had started its forward movement and it was a sharp turning-back as though the band had suddenly come into the field of vision. In an all-white cylinder (F) (an experiment used frequently to test whether individuals had any particular bias), the mosquitoes usually went straight ahead.

While the typical responses of both *Anopheles maculipennis* and *Culex molestus* females are as described above, there were always a few specimens of each species which did not conform to any fixed rule, moving at random over the arena and not moving consistently towards or away from the black. These atypical cases represent about 10% of all specimens tested.

The total number of specimens tested exceeded a hundred *C. molestus* and fifty *Anopheles maculipennis*, though records of the tracks were not kept in some cases once the general nature of the responses had been determined.

(*b*) *Narrow stripes.* Numerous trials were carried out to determine the narrowest black band which the mosquitoes could perceive and respond to. In these trials the band was almost always set at 45° towards one side or the other so that the turning response could be clearly noticed. Consistent and clear-cut responses to the bands were obtained when the width of the band was 20 mm. The width was progressively reduced during the trials and it was found that a sensitive specimen could respond to a band 5 mm. in width roughly nine times out of ten; when the width was reduced to 4 mm. there was a marked drop in the number of responses and at lesser widths the responses became quite uncertain. It appeared, therefore,

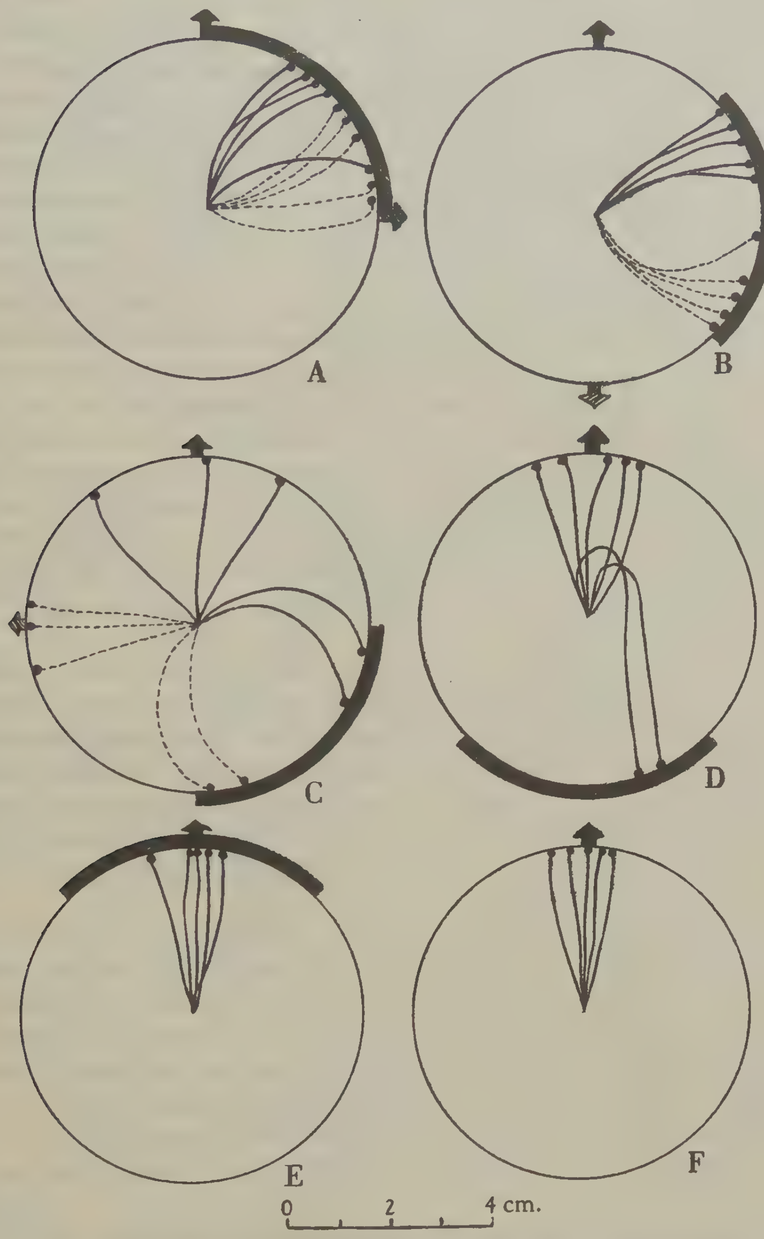

Fig. 1. *Anopheles maculipennis*. Responses to a wide dark band placed at various angles. In A, B, and C, the front edge of the dark band is at 0, 45 and 90° to one side respectively. In D the centre of the band is directly behind the mosquito, and in E directly in front. F is an all-white cylinder without any dark band. Continuous lines in A, B, and C, indicate responses when the band was to the right of the mosquito and the broken lines when it was to the left. The broad arrows indicate the initial direction of the mosquitoes at the centre of the arena: the solid arrow relates to the continuous lines and the cross-shaded arrow to the broken lines.

that at a distance of 3·5 cm. a 5 mm. wide vertical stripe, subtending an angle of about 8°, represented approximately the limit of visual acuity of the mosquitoes.

(*c*) *Two equal bands.* A large number of tests were carried out with both *A. maculipennis* and *Culex molestus* in a cylinder with two black vertical bands of about 20 mm. width. The bands were so placed that their two extreme edges were about 90° apart from one another on the circular wall. The position of the mosquito was so adjusted that it faced directly between the two bands. Fig. 2 illustrates the tracks of typical responses of *Anopheles maculipennis* and *Culex molestus* when the cylinder was adjusted so that the mosquito was at its centre. Almost identical responses were obtained when the distance between the mosquito and the bands was increased by adjusting the position of the mosquito very near the wall opposite to the bands. The response was to one of the dark bands and not to the area between them. The direction of movement of the specimen appeared to be decided almost at the start. There was no case in which the mosquito changed over to another band after having set a course towards one of them, but there were a few cases in which the mosquito normally going to one of the bands went in between the two but on reaching the extreme edge of the arena turned towards one. (Two such responses are illustrated in Fig. 2B.) When similar trials were carried out in a large cylinder of 15 cm. diameter with two black bands of 2·5 cm. width, the results were exactly the same and the mosquitoes could detect, and move towards, the black bands from a distance of 12 cm.

(*d*) *Two bands of different widths.* A few trials conducted with bands of different widths are illustrated in Fig. 2. In C and D the two bands, 1·2 and 3·5 cm. wide, were so placed that the centre of the intervening white area was facing the mosquito and in E and F the narrower band was directly in front while the broader band was to one side. In both cases the movement was directly towards one of the bands; sixteen responses were towards the narrow band and twenty-five towards the wider one. In one instance only did the specimen change over to the wider band after nearly reaching the narrow one.

(*e*) *Unilaterally blinded specimens.* Several specimens of both species were unilaterally blinded by complete occlusion of one eye with a light coat of shellac mixed with indian ink. Such insects, when enclosed in a cylinder with an all-white wall, performed typical circus movements turning towards the blinded side. Fig. 3A illustrates a typical track of an *Anopheles maculipennis* blinded in the right eye and Fig. 3B a similar one of *Culex molestus* blinded in the left eye. Only one track in each case has been drawn to avoid confusion of lines.

When enclosed in a cylinder with a single dark band, 12 mm. wide, the circus movement continued until the band came into the field of vision of the functional eye; the mosquito then turned sharply towards the dark band (Fig. 3C, D).

(*f*) *Presentation of a second band to a mosquito moving towards another.* A large cylinder of 15 cm. inner diameter made it possible to carry out a new type of test. By fixing a paper screen to the wall in a suitable position it was possible to arrange the mosquito so that only one of the bands was visible to it (Fig. 4). Once the mosquito started to move towards the visible band, the second band came into its

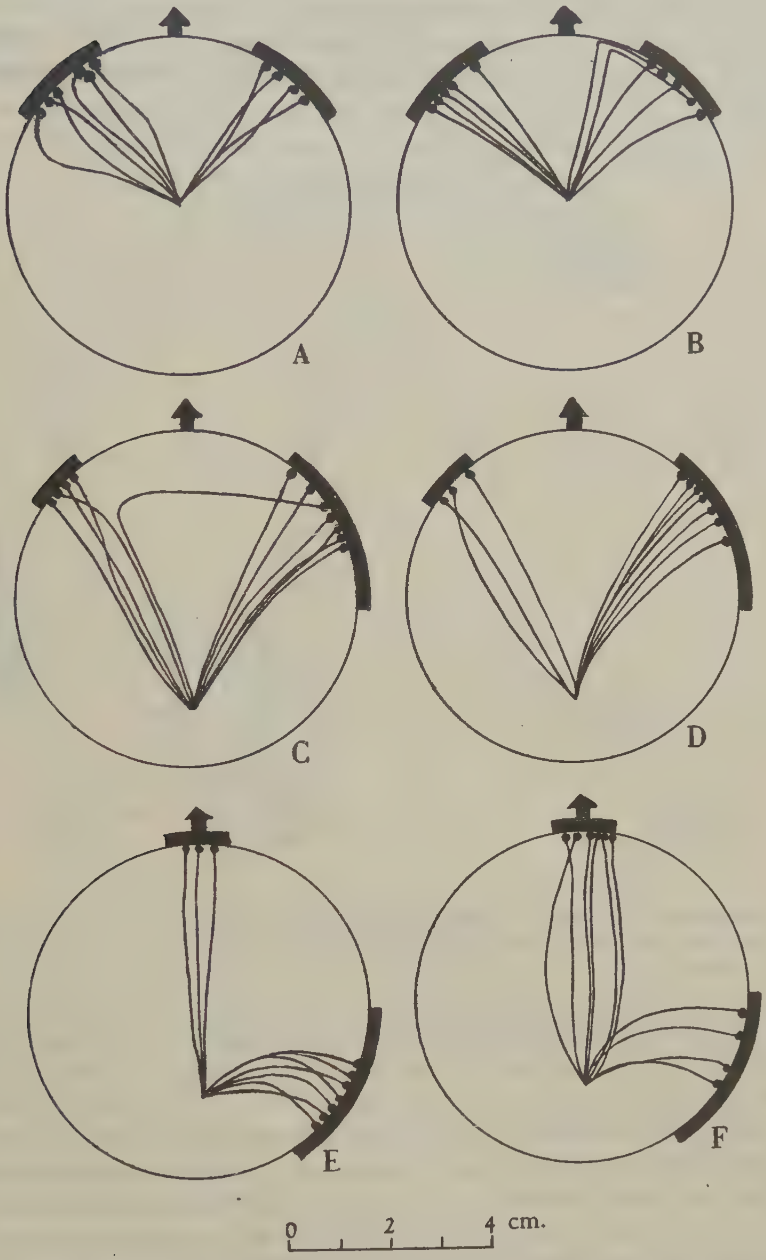

Fig. 2. A, C, and E, *Anopheles maculipennis*; B, D, and F, *Culex molestus*. A and B, responses to two equal bands with the mosquito initially facing in between them. The two responses (in B) between the bands were exceptional. C and D, the responses to two unequal bands with the mosquito originally facing between them. The single case of switching over from one band to the other in C was exceptional. E and F, responses to unequal bands one of which was directly in front.

view as soon as it passed the edge of the screen. A few of the responses are illustrated in Fig. 4. It will be noticed that quite often the sudden appearance of a new band at one side caused an immediate turn towards it.

The following observation was made with the ordinary cylinder having either one band or two equal bands. The mosquito was allowed to start moving towards one band and the cylinder was then set in rotation. So long as the rotation was slow the mosquito continued to face the same band, but if the rotation was rapid it failed to do so.

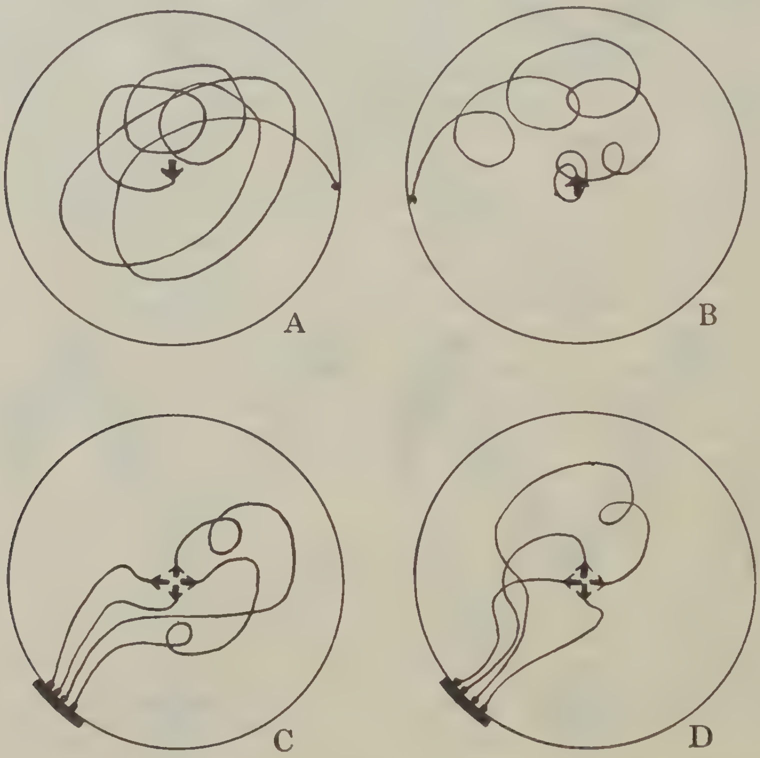

Fig. 3. Circus movements. A and C, *Anopheles maculipennis*; B and D, *Culex molestus*. A and B in uniform white light and C and D in the presence of a narrow dark band. Only one response drawn in A and B; four tracks in each of C and D. The broad arrows indicate initial position.

(*g*) *Black cylinders with white bands.* Tests were made in an all-black cylinder with a white band, 30 mm. wide, to see if movement to black described in the above experiment was due to contrast. If so, the white band on a black background should attract the mosquitoes as readily as the black one on a white background. In not a single case was movement or even a slight curvature towards the white band observed. When two white bands were presented the movement was towards the black intervening area. If the white band was directly in front, the mosquitoes curved away from it.

(2) *Experiments with artificial and natural light before and after dusk*

All the experiments described so far were carried out during daytime but several similar experiments carried out after dusk failed to reveal any change in the type of responses. In all such experiments the mosquitoes had been kept in natural light in the laboratory and therefore if there was any reversal of reaction due to natural causes it might have been expected at dusk. It was thought that the continued negative phototaxis even after dusk might be due to the strong artificial light used and therefore tests were carried out (1) with progressively reduced intensities of artificial light, finally almost reaching the limit of visibility; and (2) with the natural light of the evening.

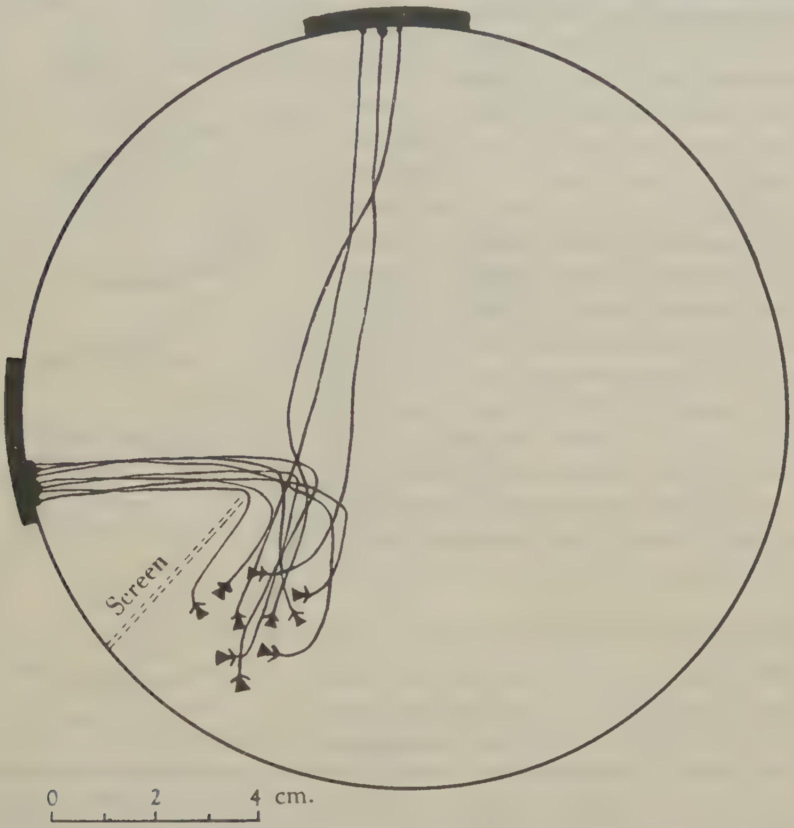

Fig. 4. *Culex molestus.* Response when a new band comes into view while already moving to another band. Arrows indicate initial position.

Except for a few trials with subdued artificial light coming from below, these experiments could not be carried out in a white arena illuminated from below and therefore the experimental conditions were slightly modified. The inner surface of the cylinder was covered with black paper except for a narrow vertical slit of 8 mm. The floor of the arena was of black matt paper and to the top of the cylinder was fixed a long narrow black paper cone, about 25 cm. in height, with an aperture at the end for viewing the arena from above, without allowing any other light to fall on the arena. The electric lamp was placed about 30 cm. to one side of the stage so that it threw a horizontal beam of light at the level of the stage and the light penetrated the arena through the vertical slit in the black paper. A mosquito was put on the stage, the cylinder inverted over it and the light switched on. In this black arena with a horizontal beam of light, the mosquito was barely visible except when directly within the beam. It was impossible to adjust the position of the mosquito in relation to the illuminated slit and therefore whatever position it assumed in relation to the beam was recorded. It was noticed that all but one of over thirty specimens tested moved away from the beam towards the darker part of the arena. Fig. 5A illustrates typical responses of a specimen of *Culex molestus*, and *Anopheles maculipennis* was found to behave in exactly the same manner. Even a considerable reduction in the intensity of the light failed to produce a change in the responses. When the experiment was carried out using the natural evening light after dusk, the responses were exactly as in artificial light. The tracks of the single exceptional *Culex molestus* which showed clear responses towards light are illustrated in Fig. 2B.

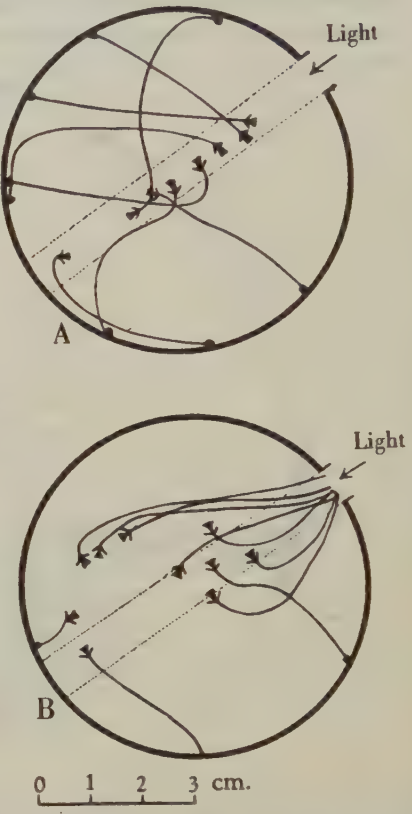

Fig. 5. *Culex molestus*. Responses in an all black arena with a horizontal beam of artificial light coming from one side. The zone of the horizontal beam marked by two parallel dotted lines. Arrows indicate initial position. A, typical responses; B, the responses of a single exceptional specimen.

(3) *Experiments in natural light in the open room*

(*a*) *With wings removed.* Numerous tests with over twenty specimens of each species were made without using the special apparatus described above. The mosquitoes were tested on a white sheet of paper placed on the laboratory bench near a large window. Individuals were placed on the sheet of paper and the tracks made by them recorded. The tests were first made in the middle of the afternoon in bright light and the same specimens were tested at or after dusk in the same manner, at a time when the free-flying mosquitoes in the room were attempting to escape through the window. Fig. 6 shows the tracks made by a specimen of *Anopheles maculipennis*, before and after dusk. It is very clear that even in the subdued light of dusk specimens whose wings have been cut off continue to curve away from light. This negative phototaxis was very clear in most of the cases. In a few individuals the responses were indeterminate and the direction of movement did not seem to be related to the direction of the light, but in the majority the responses were consistent and movement was away from the light, even after dusk.

During these experiments, when the mosquitoes were walking away from the window, a strong beam from a flashlight was often turned on the mosquito from several angles. Invariably the mosquito immediately turned away from the beam.

(*b*) *With undamaged wings.* Since it was considered probable that the consistent negative phototaxis of the mosquito with the wings cut off might have been due to some damage to an undetermined sensory centre, a few tests were carried out with specimens in which (1) the wings were stuck together with a small drop of shellac placed at their tips, and (2) with the wings simply tied together with a piece of hair. Both these types continued to exhibit the movement away from light, before, at and after, dusk.

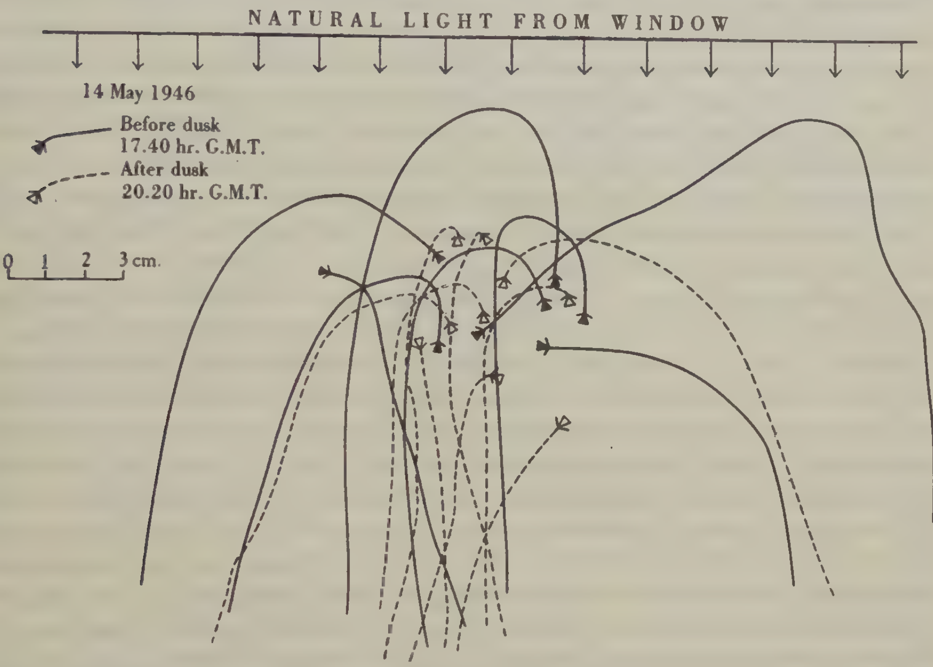

Fig. 6. *Anopheles maculipennis.* Responses of the same specimen before and after dusk to natural light coming from a window. Heavy arrows and continuous lines indicate initial position and responses before dusk, white arrows and broken lines indicate the same after dusk.

(*c*) *Experiments in Petri dishes with a darkened section.* A variation of the above experiments, to reduce physical handling of the mosquitoes to a minimum, was tried as follows: Eight to twelve specimens of *Culex molestus* with the wings removed were put into a covered Petri dish and placed on a black sheet of paper on the laboratory table. The Petri dish was covered by a bigger shallow glass dish, half of which was lined by black paper both at the top and sides so that one-half of the Petri dish became completely shaded. The specimens invariably walked towards the darker half of the Petri dish and settled there. After 15 min. the Petri dish was very gently rotated so that the section with mosquitoes came to lie in the full light.

In a few seconds the mosquitoes began to move again towards the shaded half and collected there. This was repeated several times with similar results. The experiments were repeated both before and after dusk without any difference being noted in behaviour.

DISCUSSION

The important points arising from the above observations and requiring a few words of elaboration are (1) the consistent negative phototaxis without any indications of a reversal of response at or after dusk, and (2) the mechanism or orientation.

The total absence of any reversal of the direction of response to light at dusk was an unexpected and striking discovery. Hundertmark (1938) has suggested, on the basis of certain laboratory experiments with flying mosquitoes, that such a reversal does occur. This matter certainly needs further experimental investigation, but the well-known fact that mosquitoes are 'attracted' to the exits of shelters at dusk, irrespective of their physiological state, has been clearly established in recent studies (Eyles & Bishop, 1943, Viswanathan, Ramachandra Rao & Ramarao, 1944).

Possible explanations of this behaviour are: (1) that the phototactic response is reversed in sign; (2) that the mosquitoes show positive phototaxis in weak light, up to such intensities as occur in the evenings and that no reversal of reaction is involved (Weiss, 1913); (3) that the behaviour is due not to taxis but to kinesis.

Be that as it may, it is established that flying mosquitoes tend to move towards the bright exits of their shelters at dusk and it is therefore of considerable interest to find that mosquitoes rendered flightless always move away from light however weak it may be, even in the natural twilight of the evening. It could not have been due to damage to any undiscovered sense organs located on the wings or connected with the wings because the same type of response occurs in individuals with wings which are lightly tied up together with a piece of hair and are otherwise quite undamaged. Considering also the fact that responses are similar in specimens of varied gonotrophic conditions, it seems highly probable that the movement of flying mosquitoes towards light at dusk is a process which involves the use of wings, and is a phenomenon bound up with the spontaneous flight activity. This aspect of the problem will be dealt with at greater length in another paper in connexion with certain studies on the phases of the diurnal cycle of activity in mosquitoes.

It has to be considered whether this permanent negative phototaxis of 'wingless' specimens could be explained as an 'escape' response of an 'excited' individual or as a shelter-seeking response of a disabled one. All the specimens used in these experiments were, in a way, disabled individuals because none of them could use the wings. Some of them must certainly be regarded as 'excited' because of repeated handling and knocking about, but quite a few, such as those used in the Petri-dish experiments, were practically undisturbed. Among the latter the only handling involved was a very gentle and slow rotation of the dish to bring the originally shaded half into the light and the lighted half into deep shade. After this gentle change, the mosquitoes slowly began to move and then to walk back to the shaded part. These responses, observed in the total absence of physical disturbances of any kind, were exactly similar to the responses of mosquitoes which were thrown about, often with some force, in the arena. This seems to indicate that the movement of walking mosquitoes towards darkness does not depend upon 'excitation'. In any case the flying mosquitoes, in an excited state, are supposed to fly *towards* light as an immediate response and not towards darkness (Polezhaev, 1936; Kennedy, 1940). Inability to use the wings seems to lead to inhibition of certain types of activity, a problem of some physiological interest. It may be that the lack of wings, so essential for normal activity, always induces mosquitoes to look for shelter and, therefore, instinctively to move towards darkness.

As to the mechanism of orientation, it may be stated that the present studies broadly confirm the observations on flying mosquitoes made by Kennedy (1940). It is very difficult, however, to determine whether the movement towards the black bands is tropotactic or telotactic. Fraenkel & Gunn (1940) have made it clear that the same animal may be tropotactic and telotactic at the same time. As few animals can see directly behind them (and the mosquito certainly cannot see directly behind it; for example, a black band, 10 mm. wide, placed directly behind it in the cylinder at a distance of 3·5 cm. is practically invisible to it), there can be no negative phototelotaxis. Even when the edge is at 135°, as has been shown in the experiments, only in a few cases does the movement to black take place. It is not possible to regard the movement to black exhibited by the mosquitoes as telotaxis, unless, of course, we believe in the existence of 'skototaxis' as a definite type of orientation. But on the evidence at present available the behaviour can be explained equally convincingly as negative phototropotaxis. Unfortunately the circus movements performed by unilaterally blinded specimens in uniform light such as described above can be performed telotactically as well as tropotactically and therefore are not likely to be useful in distinguishing between these two types of response. The movement to one of the dark bands when two are presented and not to a point in between them has also been explained by Fraenkel & Gunn (1940) as due to negative tropotaxis. The only semblance of telotaxis is the response of a mosquito (Fig. 2C) already moving towards one of two visible dark bands, deflecting its course towards the other. The conditions are quite different when a new band suddenly comes into view as in Fig. 4. Whether this can be explained as an incipient form of vision is a matter requiring further investigation. But it appears to be almost exactly like the responses found by Kennedy when he observed that *Aëdes* was attracted to and followed stripes which were moving, abandoning stationary ones. It is possible that to the walking mosquito already moving towards a stationary stripe straight ahead, a *new* stripe which suddenly looms into view on one side provides an illusion of a backwardly moving band and the mosquito therefore turns towards it. Once it turns and heads towards the new stripe the direction becomes fixed and the old stripe no longer influences it, just as in the case of the experiments with two bands both of which were simultaneously visible from the very beginning.

Turning now to the case of the unilaterally blinded specimen performing circus movements, we recall that when the dark band suddenly comes into the field of the functional eye the animal turns abruptly towards it. How far this type of behaviour can be fitted into the general idea of a negative phototropotaxis is not clear. If tropotaxis is a result of a balance of stimulation by simultaneous comparison, then circus movements should continue even in a beam or gradient; the total ipsilateral turning effect of the blind eye of a negatively phototactic animal should continue to be greater than the opposing effect produced on the functional eye by a narrow dark band, because the latter can affect only a small proportion of ommatidia of the functional eye and not all of them. Here again the effect of the sudden appearance of the band in the view of the functional eye may result in an illusion of movement leading to orientation towards it.

There is another important point to mention regarding the region of the dark bands towards which the movement takes place. In typical cases when the band was either to the left or the right of the specimen, the movement involved first turning towards the appropriate side and then walking towards the black band. In such a movement the first point of the dark band to be reached was usually the extreme edge. The mosquito sometimes walked a few millimetres from the edge of the black band towards its centre, then climbed the wall and stayed there. But in many cases the movement proceeded further than that and the mosquito climbed the black wall at about the middle section of the band. But since in a few cases the mosquito remained near the edge it was necessary to make further experiments to discover if there was any evidence of photohorotaxis (Kalmus, 1937). Accordingly, many observations were made in the cylinder with the 90° black band, placing the band in such a way that the mosquito directly faced its centre (Fig. 1E). It was noticed that the mosquitoes moved towards the middle of the band without showing any tendency to curve towards either edge. The apparent attraction of the edge when the band was placed to the side was simply due to the fact that the mosquitoes had to move along a curved track towards the band and so reached it first near the edge, where they then remained. In the experiments with two bands it was also a matter of interest to observe the position at which the specimens came to rest. There was no indication of their coming to rest near the inner edge of one of the bands: had they done so the idea of a negative phototropotaxis would have received further support. On the whole the observations do suggest that the visual orientation of the mosquito can be regarded as due to a negative phototropotaxis.

Unfortunately lack of time did not permit quantitative estimation of turning responses in relation to the position of a single dark band at various angles in the horizontal plane. It was noticed that when the edge of the dark band was directly in front or about 45° to left or right of the longitudinal axis of the head the responses were perfect, that is the movement to the black was nearly always completed. But when the edge lay at about 90° to one side the movement to black did not take place quite so often, and when the edge of the band was 135° towards one side the movement to black was still less common. In a few trials conducted in a cylinder with a band of only about 10 mm. width, very similar differences in the responses were noted. These suggest that the anterior ommatidia are more sensitive to the black band or perhaps initiate turning movements to black more strongly than lateral ommatidia, a view which is in agreement with Kennedy's (1940) postulation. Of course, if the band is directly behind them the mosquitoes cannot see it, and therefore are not likely to respond to it. But when it is anywhere from 0 to 110° from the front the number of ommatidia stimulated must be more or less the same, because at this plane the visual angle of the eye is very nearly 225° all round and 110–115° on either side. It might be possible with this new method of study with wingless mosquitoes to make a complete reflex-map of the eye by using a small dark spot of adequate dimensions and exposing it to the mosquito at all possible angles so as to affect particular groups of ommatidia.

The variabilities in behaviour of individual specimens have already been mentioned. Only about one in ten of the specimens failed to show any sort of consistent response; the reason for this is obscure. Fraenkel & Gunn (1940) (pp. 292–3) have stressed that the future behaviour of a particular individual is always unpredictable with a 100% assurance, but what the majority of a given population will do can be foretold reasonably correctly. Exactly the same thing occurs in the mosquitoes so far examined. There must always be unusual individuals in any group which may be regarded as extreme variants of a normal distribution. An analytical study of their behaviour would contribute to an understanding of the behaviour of the normal ones. In the present series of observations, however, the behaviour of the atypical forms has been extremely inconsistent, and they have shown no regular pattern of behaviour.

SUMMARY

Most females of *Anopheles maculipennis* and *Culex molestus* which had been rendered flightless by removal of wings or by sticking the wings together, tended to move towards a black vertical band when it was presented to them in an arena uniformly illuminated from below. The narrowest width of the band to which they could consistently respond was 0·5 cm. at a distance of 3·5 cm. corresponding to an angle of about 8°.

When the edge of a wide black band was exactly in front of the mosquito or at about 45° to one side the movement towards it was consistent, but when the band was placed more and more posteriorly consistency of the responses progressively decreased.

When offered simultaneously two black bands of equal or different widths the movement was to one of them. If a new band came into the view when the mosquito was already moving towards another, a turning response towards the new one took place very frequently.

Unilaterally blinded specimens performed circus movements in uniform light. When a narrow black band came into the visual field of the functional eye there was an immediate turning response towards it.

The negative phototaxis continued even at and after dusk at a time when flying mosquitoes were showing natural movement towards the evening light. Reduction of intensity of illumination and also the natural fading of the light at dusk both failed to bring about a reversal of response. For some undetermined reason mosquitoes rendered flightless always tend to move away from light.

The mechanism of orientation seems to be in conformity with current ideas of negative phototropotaxis. No evidence of a photohorotaxis was secured.

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THE INFLUENCE OF TEMPERATURE AND HUMIDITY ON INSTAR LENGTH IN *CALANDRA GRANARIA* LINN.

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(With Five Text-figures)

In an earlier paper, Eastham & McCully (1943), the results of an investigation into the effects of temperature and humidity on the oviposition responses of *Calandra granaria* were published. There the effects of environment on the multiplication of cultures of *Calandra* were recorded in so far as that increase depended upon the rate of egg-laying, number of eggs laid and the length of ovipository life of females. An attempt is made here to extend that information, not only by showing how environment determines the total duration of a developmental period, but how it affects each separate instar of that period from the egg to the emergence of the adult from the pupa.

Works making such a detailed approach to the problems of environmental control of insects are few and in view of the great labour involved in the present investigation (we have examined some 50,000 grains of wheat) we are not surprised that this is so.

TECHNIQUE

All the weevils used as parent stock in these experiments were reared on soft English wheat at 25° C. and at room atmospheric humidity which was uncontrolled but which varied little from a relative humidity of 70%. From these stocks females were isolated into gauze-covered glass tubes $2 \times \frac{1}{2}$ in. To each female was given a single grain of wheat which had been previously acclimatized in its water content to the atmospheric humidity of the experiment about to be made. Each day the wheat grain was removed and replaced by another, the removed grains being then placed in shallow glass dishes, inside screw-topped glass jars, capacity 750 c.c. Inside each of these jars the atmospheric humidity was controlled by solutions of KOH of known specific gravity (Buxton & Mellanby, 1934). The ovipositing females providing the growth stages in the grains of wheat were kept at 25° C. and at a relative humidity of 70%, conditions about the optimum for this purpose. Each grain of wheat was therefore kept for 1 day only under conditions different from those to which it was subsequently subjected in the course of experiment. The grain was in all cases first acclimatized to an appropriate atmospheric humidity, as in the experiments on oviposition by Eastham & McCully (1943), namely, by spreading it out on shallow trays in different controlled relative humidities of air for a minimum period of 30 days. In this way the water content of the grain was brought into stable relation with the moisture of the air of its own experiment.

The moisture content of the grain thus attained for each R.H. was deemed to be similar to that used in experiments on oviposition (Eastham & McCully, 1943), and given in the following table:

R.H. of air (%)	80	70	60	50	40
Water content of grain (%)	15·98	13·82	13·26	11·29	10·56

The grain in which eggs had been laid was kept under the following conditions of constant temperature, 15, 20, 22·5, 25, 27·5 and 30° C. At each temperature we had a range of atmospheric humidities from 40 to 80% at intervals of 10%, with the exceptions that at 15° C. the lower and higher R.H. of 40 and 80% were omitted and at the temperatures 20 and 30° C. the R.H. of 80% was also omitted. At the lowest temperature the low humidity of 40% was ineffective, no development taking place, whereas at the highest temperature mould growths interfered seriously with the cultures in all our attempts to employ the 80% R.H. From the beginning of each experiment set up, grain was dissected so as to discover the stage or stages of development reached by the contained organisms. Each day enough grains were dissected to obtain twelve living stages. These were fixed and stored so that later they could be examined. The daily incidence of all stages of development we found were then plotted as a series of overlapping curves of distribution. For each combination of temperature and humidity a separate graph was drawn, the length of each instar being measured in days as between the points of intersection of its own curve with those of preceding and succeeding instars. Fig. 1. is an example of such a graph and shows the method used to deal with such discrepancies as are inevitable to the random-sampling method which was employed.

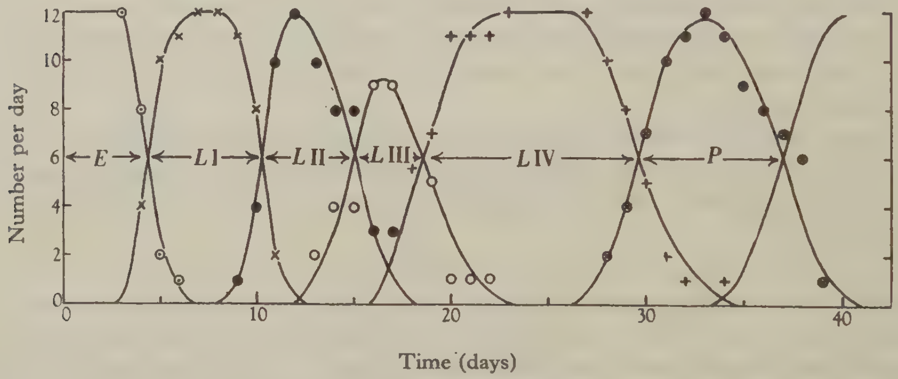

Fig. 1. The development of *C. granaria* at 25° C. and 70% R.H. illustrating the method used to determine the duration of the various stages under each set of experimental conditions. Composition of daily sample dozens indicated as follows: egg —⊙—; larva I —×—; larva II —●—; larva III —○—; larva IV —+—; pupa —⊗—. Points of intersection of interpolated curves represent average times of moults; distances between successive points of intersection thus represent average durations of the several stages. *E*=egg; *L* I, II, III and IV=first, etc., larval instars; *P*=pupa.

EXPERIMENTAL RESULTS

The data thus obtained from the various graphs is summarized in Table I and Fig. 2. For each temperature the separate columns of the histogram give the total days taken for development from egg to adult emergence at each of the percentage

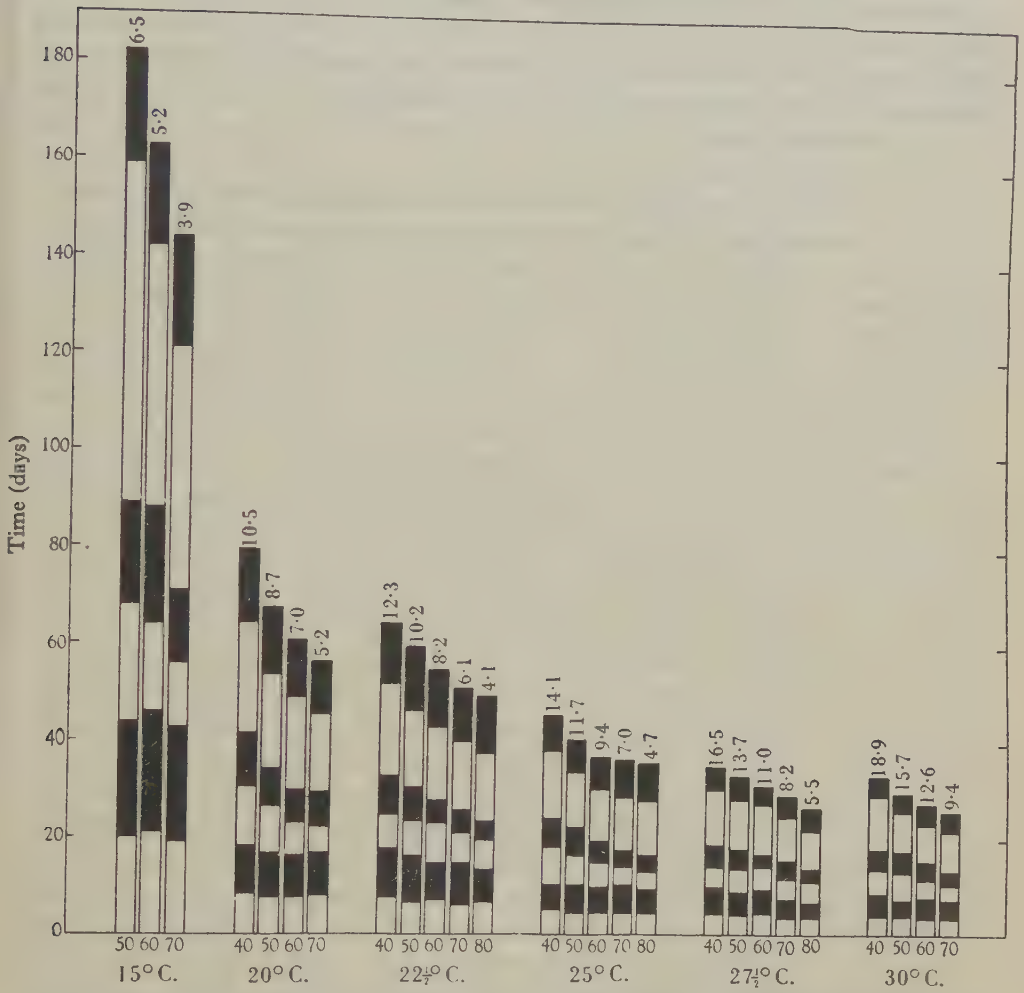

Fig. 2. Histogram showing the duration of all stages in development of *C. granaria* from egg to pupa at each combination of temperature and atmospheric humidity employed. Columns grouped according to temperature; figure at foot of each column indicates atmospheric humidity as percentage R.H., corresponding S.D. in mm. mercury being shown at apex of column. Alternate black and white bands represent successive stages in development beginning with egg (white) at the base and finishing with pupa (black) at the top.

R.H. indicated at the foot of each column. There being often a delay in emergence of the weevil from the grain after the animal has freed itself from the pupa, it should be understood that 'adult emergence' in this case means emergence from the pupa and not from the grain. Since with differences of temperature any given R.H. corresponds to important differences in saturation deficiency (S.D.) of the air, we have placed above each column the S.D. in mm. of mercury which applies to it. It then becomes possible not only to compare the effects of changes in S.D. at one temperature, but also to attempt the determination of the effect of changing temperature under constant moisture conditions. Each column in Fig. 2 is divided into alternate white and black bands representing the succession of developmental stages, starting with the egg stage at the bottom and ending with the pupa (black) at the top. We can, therefore, extend the investigation from the cycle as a whole to a treatment of each instar.

Table 1. *The duration in days of each developmental stage under the various combinations of temperature and relative humidity employed.*

Stage	Grain % H_2O	R.H. %	15° C.	20° C.	22·5° C.	25° C.	27·5° C.	30° C.
Egg	10·5	40	—	8·5	7·5	5·0	4·5	4·0
	11·3	50	20·0	7·5	6·5	4·5	4·0	4·0
	13·3	60	21·0	7·5	7·0	4·5	4·5	3·5
	13·8	70	19·0	8·0	6·0	4·5	3·5	3·5
	16·0	80	—	—	6·5	4·5	3·5	—
Larva I	10·5	40	—	10·0	10·5	5·5	5·5	5·0
	11·3	50	24·0	9·5	10·0	6·0	5·0	4·5
	13·3	60	25·0	9·0	8·0	5·5	5·0	4·5
	13·8	70	24·0	9·0	9·0	6·0	4·0	4·0
	16·0	80	—	—	7·0	5·0	3·5	—
Larva II	10·5	40	—	12·0	7·0	7·5	4·0	4·5
	11·3	50	24·0	9·5	7·0	6·0	4·5	4·5
	13·3	60	18·0	6·5	8·0	5·0	4·5	3·5
	13·8	70	13·0	5·5	6·0	3·5	4·0	3·0
	16·0	80	—	—	6·0	3·5	4·0	—
Larva III	10·5	40	—	11·0	8·0	6·0	5·0	4·5
	11·3	50	21·0	8·0	7·0	6·0	4·5	4·5
	13·3	60	24·0	7·0	5·0	4·5	3·0	4·0
	13·8	70	15·0	7·0	5·0	3·5	4·0	3·0
	16·0	80	—	—	4·0	3·5	2·5	—
Larva IV	10·5	40	—	23·0	19·0	14·0	11·0	11·0
	11·3	50	70·0	19·0	16·0	11·0	10·0	8·5
	13·3	60	54·0	19·0	15·0	10·5	10·0	7·5
	13·8	70	50·0	16·0	14·0	11·0	9·0	8·0
	16·0	80	—	—	14·0	11·0	8·0	—
Pupa	10·5	40	—	15·0	12·5	7·5	5·0	4·0
	11·3	50	23·0	14·0	13·0	7·0	5·0	4·0
	13·3	60	21·0	12·0	12·0	7·0	4·0	4·5
	13·8	70	23·0	11·0	11·0	8·0	4·5	4·5
	16·0	80	—	—	12·0	8·0	5·0	—

I. *Effects of temperature*

Examination of the data presented in Table 1 shows that both temperature and atmospheric humidity have considerable influence on the duration of development. In this section the attempt will be made to extract from the data information as to the effect of the single factor of temperature on the period of development. For this to be possible we must be satisfied that insects reared at different temperatures have been subjected to the same moisture conditions. The problem is by no means simple since it concerns the total water relations of the insect.

The essence of the problem would seem to be ensuring that the state of desiccation of the insects is maintained constant over the whole range of temperatures employed. This in turn means ensuring that equilibrium is maintained between the input and output of water—the input water consisting partly of food water, partly of metabolic water, the output water of water lost by evaporation and any water lost with the excreta. The problem is still further complicated by the fact that all these water exchanges are themselves influenced to varying degrees by changing temperature, since a rise in temperature may be expected to lead to an increased rate of feeding and thus to a more rapid intake of food water, and also to an increased rate of production of metabolic water. On the output side, Ramsay (1935) has shown that the rate of evaporation rises with an increase in temperature even under conditions of constant saturation deficiency. It is clear, therefore, that there is no simple means of subjecting the insect to a predetermined set of moisture conditions. If, for instance, we employ a common S.D. at various temperatures we are subjecting the insect to an approximately constant rate of water loss by evaporation while the intake and production of water are varying If, on the other hand, we employ a common R.H. at the different temperatures we ensure a constancy of food water whilst the rate of production of metabolic water and rate of water loss will vary with the temperature. It is worth noting here that the common practice of employing a single R.H. at different temperatures as a means of controlling the humidity, e.g. Ewer & Ewer (1942), is probably the best method of applying a predetermined condition. Production of metabolic water and rate of water loss both vary directly with temperature and calculation shows that where Q_{10} is of the order of 2, these two processes vary in step with one another, and hence cancel one another in their effect on the water balance of the insect. Discrepancies are, however, bound to occur at the upper and lower limits of the viable range of the insect, where the metabolic rate of the insect is depressed, or where the van't Hoff coefficient differs widely from 2.

The method we have accordingly adopted is as follows. Working on the primary assumption that the essential effect of temperature is mainly to vary the rate of the insect's metabolic processes we infer that, provided the temperature only is subject to variation, then the 'water intake' during the whole, or any particular part of the developmental cycle will itself not vary, although the *rate* at which this takes place will do so. If we ensure that during the growth period, whatever its length, the total water loss is similarly unchanged we can justifiably infer that the water balance of the insect remains undisturbed, whatever the temperature to which it is exposed. Our aim has therefore been to make comparison of the developmental times of insects which, though exposed to different temperatures have nevertheless been subjected to the same overall water loss. Since this has involved the comparison of insects feeding on grain with different water contents, and since also our calculations regarding water loss are based entirely on the known atmospheric humidities applied to the insects, two further assumptions have necessarily been made, the validity of which must be examined. The first is that the water lost with the excreta is negligible in comparison with that lost by evaporation, the second that the difference in water content of the grain at the different humidities is of little significance when considering the 'input water' as a whole. On neither point have we any direct information with regard to *Calandra*. In view of the well-known water-conservation powers of insects, particularly of those feeding on dry substrates, we feel justified, however, in making the first assumption. With regard to the second, the work of Fraenkel & Blewett (1944) may be cited. These authors, working with three different stored product insects, *Ephestia*, *Tribolium* and *Dermestes*, found that even under optimum conditions the insects respired not less than five-sixths of the dry weight of the ingested food, and frequently a much higher fraction. If we assume that the metabolic processes of *Calandra* are of the same order, i.e. that not less than five-sixths of the dry weight of the ingested food is used as fuel, it does not appear that the water content of the grain is of any great importance. For the composition of English wheat, according to the data of McCance & Widdowson (1946) is—water 15%, non-utilizable material 8%, protein plus fat 11% and the remaining 66% starch and dextrins, the bulk of the available material being thus carbohydrate. A minimum figure for the production of metabolic water is obtained by calculating the whole of the available material as polysaccharide, in which case any given quantity will yield five-ninths its weight of water (since 162 g. starch produce 90 g. of water). Hence, if we assume that five-sixths of the dry weight of the ingested food is oxidized, we find that whereas grain acclimatized to a R.H. of 40% (water content 10·5%) yields water totalling 48·2% of the original weight of grain, grain acclimatized to 70% R.H. (water content 13·8%) yields water totalling 50% of the grain weight. The difference in water yield from grain at the two extreme humidities is thus of the order of 4%. It does not therefore seem likely that under the range of relative humidities used in the present investigation the water content of the grain is of any great significance. In making the calculations which follow we have worked on the hypothesis that the rate of evaporation of water from the insect is not only directly proportional to the saturation deficiency of the atmosphere (Mellanby, 1935*b*), but also to the square of the absolute temperature (Ramsay, 1935). (It may be noted, however, that since the range of temperature employed by us is small, viz. 15° C., this latter temperature effect is not of great significance. It has been incorporated in the calculations for the sake of completeness as it is clear from Ramsay's (1935) work that where a wider range of temperatures is employed this factor must be taken into consideration.)

The effect of temperature on the duration of the total developmental period may be taken as an example of the application of the method. A series of 'temperature factors' has been calculated from Ramsay's (1935) data, taking unity as the factor at the lowest temperature employed, i.e. 15° C.* The factors, which indicate the

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* Ramsay (1935) gives two expressions for calculating the rate of evaporation of water from a wet surface under two different sets of conditions. It is immaterial in our case which set of conditions is in operation since in our experiments certain of the variables in the expressions function as constants. Provided only that we regard the insect as a water-saturated system which maintains a saturated atmosphere in its immediate vicinity, both of Ramsay's expressions reduce approximately to the form

$$R \propto T^2 \times \text{S.D.},$$

increased rate of evaporation caused by a rise in temperature to an atmosphere with a fixed S.D. are as follows:

15° C.	20° C.	22½° C.	25° C.	27½° C.	30° C.
1	1·035	1·052	1·070	1·088	1·107

For each of the relative humidities (40–70%) at each of the temperatures employed (15–30° C.) an 'evaporation factor', as it will be called, has been calculated by taking the product of the saturation deficiency (taken from Buxton's data, 1931) and the respective 'temperature factor'. These 'evaporation factors' are shown in Table 2.

Table 2

%	15° C.	20° C.	22½° C.	25° C.	27½° C.	30° C.
40	8	11	13	15	18	21
50	6·5	9	11	12·5	15	17·5
60	5	7	8·5	10	12	14
70	4	5·5	6·5	7·5	9	10·5

Finally, the developmental times at the various combinations of temperature and R.H. have been multiplied by the corresponding 'evaporation factor'. Both the developmental times and the products, developmental time × evaporation factor (the latter in heavy type) are shown in Table 3. These products are taken as indices of the total water loss by evaporation, and hence, if comparison is to be made of insects subjected to the same water loss, we must select those having the same product. It is apparent from the table that the products range from 273 (at 70% R.H. and 30° C.) to 1183 (at 50% R.H. and 15° C.) but that only a short range is common to all six temperatures, viz. 575–630. If we are to compare insects at all the tempera-

Table 3

%	15° C.	20° C.	22½° C.	25° C.	27½° C.	30° C.
40	— —	79·5 **874·5**	64·5 **838·5**	45·5 **682·5**	35 **630**	33 **693**
50	182 **1183**	67·5 **607·5**	59·5 **654·5**	40·5 **506·5**	33 **498**	30 **525**
60	163 **815**	61 **427**	55 **467·5**	37 **370**	31 **372**	27·5 **385**
70	144 **576**	56·5 **311**	51 **331·5**	36·5 **274**	29 **261**	26 **273**

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where R = rate of evaporation, T = absolute temperature, S.D. = saturation deficiency of atmosphere to which the insect is subjected. Hence, at a given saturation deficiency, the ratio of the rates of evaporation, R_1 and R_2, at two different temperatures, T_1 and T_2, is given by $R_1/R_2=(T_1/T_2)^2$. By arbitrarily fixing one of the temperatures it is thus possible to evaluate a series of 'temperature factors' for other temperatures which represent the ratio of the rate of evaporation at the variable temperature to the rate at the fixed temperature. The lowest temperature we employed, viz. 15° C. (=288° A.), has been used in our calculations as the fixed temperature and the fraction $(T/288)^2$ evaluated for each of our other experimental temperatures.

tures employed we must therefore select a product within this range. For our purpose the highest figure—630—has been chosen, this being equivalent to the moisture conditions at 40% R.H. at 27½° C. To obtain the developmental times at other temperatures corresponding to the product of 630 some interpolation is necessary. Thus, for instance, at 15° C. 630 falls between the products at 60 and 70% R.H. and corresponds approximately to a R.H. of 68% and a developmental time of 148 days. A similar calculation has been made for each of the other temperatures. The developmental times at the various temperatures under equivalent moisture conditions are then:

	15° C.	20° C.	22½° C.	25° C.	27½° C.	30° C.
T (days)	148	68·5	59	44	35	32
$1/T$	0·0068	0·0146	0·0169	0·0227	0·0286	0·0313

These results have been expressed graphically together with the rates of development ($1/T$) in T (Fig. 3). As may be seen the rate curve is approximately linear (though more accurately is probably a flat sigmoid) and the time curve therefore approximately hyperbolic. We have clearly been dealing only with the central region of the 'vital zone' of development (vide Uvarov, 1931), i.e. 15° C. is some distance above the developmental threshold and 30° C. is some distance below the upper critical temperature. Extrapolation of the rate curve gives a theoretical zero of development of 11° C.; the actual zero will be several degrees below this temperature.

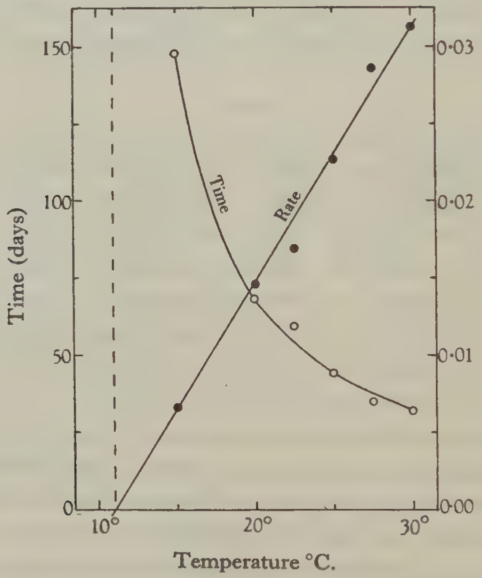

Fig. 3. Development of *C. granaria* showing the duration (T) and rate ($1/T$) of total development (egg to pupa) under moisture conditions equivalent to 40% R.H. at 27½° C. (see text for explanation). Broken line indicates the theoretical threshold temperature.

Using the method outlined above, the various stages in development have also been separately analysed for the effect of temperature. The results for the embryo, the four larval instars and the pupa are shown graphically in Figs. 4 and 5. In each case the duration of development and the rate of development ($1/T$) have been plotted against the temperature. The curves on the whole resemble those for the total development, though one or two points deserve mention. For the embryo and the four larval instars there is a fairly exact linear relation between the rate of development and the temperature and the corresponding time curves are hyperbolas. The theoretical zeros of development are in agreement, viz. *c.* 11° C., though for the second larva it is slightly lower than this figure. For the pupa, however, the rate curve is markedly sigmoidal in character and its general trend suggests that the zero of development for this stage is much lower than that of the earlier stages. These results contrast with the findings of earlier workers on other species. Glenn (1922), for instance, found in the codling moth that the threshold for the egg and larva was 10·0° C. but rose to 11·1° C. in the pupa. Blunck (1923), working with *Dytiscus marginalis*, found that the threshold of development rose in stages from 0 to 3·8° C. in passing from the egg through the several larval instars.

II. *Effects of moisture*

Reference to Tables 1 and 3 clearly indicates that humidity has appreciable effects on the rates of development. Lower moisture conditions, whether considered in terms of S.D. of the air or of water content of the food, separately or together, serve to slow down the development as a whole. The effect is obvious at each of the temperatures in the range investigated. While certain discrepancies occur in the data (Table 1) it can be seen that moisture has very different effects on the various stages. In the case of the embryo, moisture appears to have no significant effect; there is little difference in the duration of this stage at any one temperature, no matter whether the air is dry or relatively moist. The four larval instars, on the other hand, show a marked reaction to humidity though the effect is least in the first larval instar whilst the three later ones are apparently equally affected. The behaviour of the pupa is similar to that of the egg for, except at the temperature of 20° C., we can record no significant change in developmental period with changes in humidity. We cannot, therefore, by our findings on *Calandra*, confirm the results of Evans (1934) on *Lucilia sericata* where for the egg there was a linear relation between S.D. and time of development, nor can we understand that author's conclusions that more water is lost from the egg at a lower S.D. than at a higher when the temperature is raised from 14 to 22° C. Our results do, however, agree closely with those of Ewer & Ewer (1942) on the behaviour of *Ptinus tectus*. These authors found, as we did, that humidity had little or no effect on the duration of the embryonic or pupal phases (though low humidity tended to delay the hatching of the embryo) but had considerable influence on the larval development. Holdaway (1932), similarly found the duration of the egg stage and of the pupal phase of *Tribolium confusum* to be unaffected by humidity between zero and saturation at 27° C.

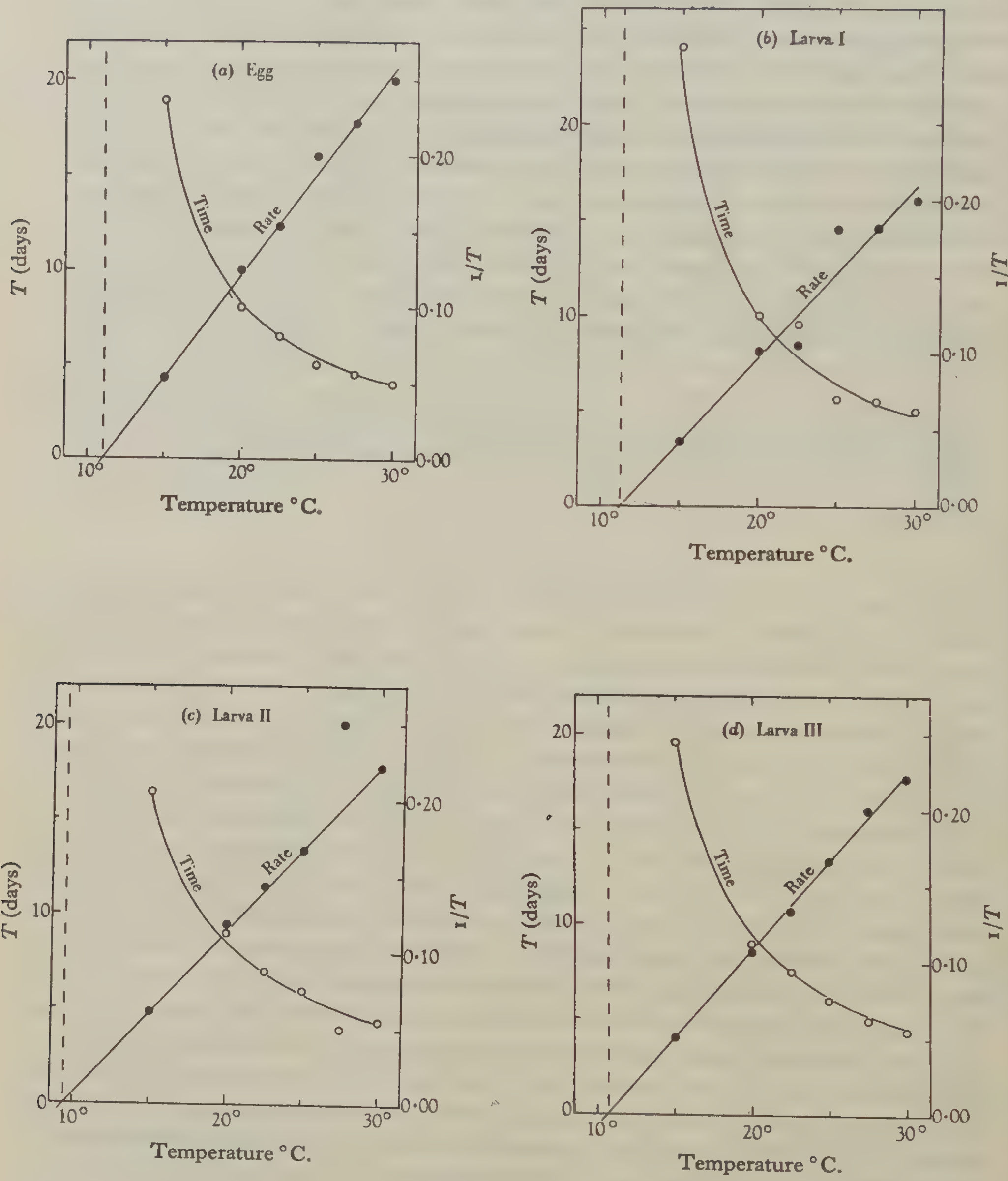

Fig. 4 *a–d*.

Figs. 4, 5. Development of *C. granaria* showing duration (T) and rate ($1/T$) of development of (*a*) egg; (*b*), (*c*), (*d*) and (*e*) the four larval, and (*f*) pupal stages under moisture conditions equivalent to 40 % R.H. at 27½° C. Note that the time scale for the fourth larval instar is half that of the other stages and that the rate scale is modified accordingly. Broken lines indicate the theoretical threshold temperatures.

The absence of response on the part of the embryo and pupa to humidity, the larval instars being affected by this factor, would appear to indicate that the burden of responsibility for influencing the larva rests with the food water and not with the atmospheric humidity, since whilst the embryo and pupa are subjected only to the atmospheric humidity, the larva being a feeding phase, is subject both to atmospheric humidity and food water. In the previous section, however, we advanced reasons for believing that the difference in the moisture content of the food at different relative humidities is of small account, since the food water forms only part of the total water available to the insect, the other and possibly the major fraction being metabolic water. If our assumption is correct, then the atmospheric humidity is mainly, if not entirely, responsible for variation in the duration of the larval instars.

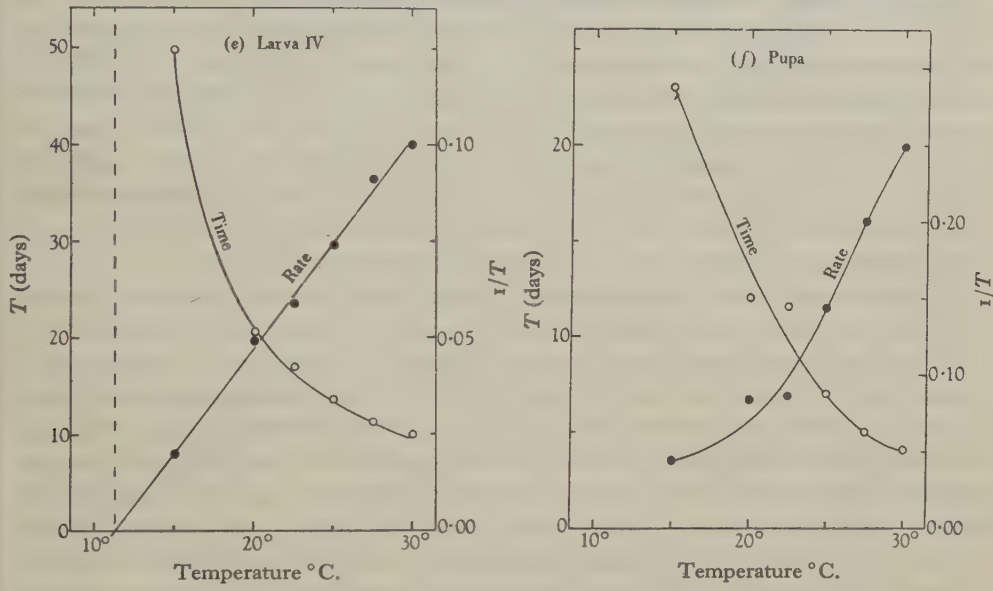

Fig. 5*e*, *f*. For explanation see Fig. 4.

How then can we explain the behaviour of the embryo and pupa? The explanation in part appears to be that the normal physiological processes of both embryo and pupa are in one important respect in complete opposition to those of the larva, for whereas the latter is a feeding and growing phase, the former two can readily be described as starvation phases. If any sort of balance is to be maintained in the ratio of dry matter to water the anabolic processes of the larva involve the locking up of water, the catabolic processes of the embryo and pupa in the freeing of water. The physiology of the larva is thus in competition with the process of evaporation whilst that of the embryo and pupa favours it. Putting it another way, if the larva metabolizes a given quantity of food material, in its case of extraneous origin, the water so produced is required for a dual purpose, partly to meet the demands of evaporation, and partly for incorporation in its own tissues. The embryo or pupa metabolizing an equivalent amount of food is drawing on its own tissues. In so doing it reduces its dry weight, which in turn releases a margin of tissue water, and this, together with the water of metabolism, is available for purposes of evaporation. The larva has thus a single water supply for which there is a dual demand; the embryo and pupa have each a dual water supply for which there is a single demand. One can readily visualize circumstances in which the larva on the one hand is suffering from a deficit of water, whilst the embryo and pupa on the other, are suffering a surfeit. We suggest that in our case the humidity conditions we have applied are centred on the optimum for the embryo and pupa but not for the larva. Hence the latter shows an obvious response to atmospheric humidity, whilst the other two phases show but little.

Other factors too may play a part in the behaviour of the embryo and pupa. In the case of the embryo, since the eggs are all derived from females in the same physiological state and are laid under identical conditions, they are all alike at the beginning of development. There is bound to be some delay before they come into equilibrium with atmospheres of different humidity, and the metabolic processes just discussed are calculated to protract this period. We believe in fact that this is the explanation of the rather small response of the first larval instar to humidity; that a sufficient balance of water is carried over from the egg to the first larva to offset the evaporating power of the atmosphere. Not until the second larval instar begins does the organism encounter the full impact of atmospheric humidity.

In the case of the pupa there exists the possibility that by some process of internal regulation the water content of the organism is adjusted, however much it has been disturbed in the preceding larva. Auber & Raymont (1944) claim to have demonstrated that this does take place on pupation in *Ephestia kuehniella* though their results have been only partially confirmed by Fraenkel & Blewett (1944). Auber & Raymont suggest that 'the regulatory process involves the oxidation of a greater weight of reserve substances during the prepupal stage of those individuals reared in the drier environment'. Buxton (1930) and Mellanby (1932) showed that at 23° C., but not at 30° C., starving *Tenebrio* larvae similarly adjusted their water contents when exposed to different atmospheric humidities by modifying their rate of metabolism. We cannot say whether such regulation occurs in *Calandra*, but that there is some profound alteration in the metabolism of the pupa is abundantly clear from our own results. For, whilst in the earlier stages Q_{10} is of the order of 2 between 20 and 30° C., though higher than this between 15 and 20° C., in the pupa Q_{10} lies between 3 and 4 over the whole temperature range from 15 to 30° C.

The problem of the reaction of the larva to humidity remains to be discussed. We may state at the outset that in our opinion this is in some way correlated with the state of desiccation of the insect, that at any one temperature atmospheres with different saturation deficiencies tend to induce different states of desiccation in the insect and that these, in some way not as yet explained, retard the processes of growth and development to varying degrees. The reasons for this conclusion are as follows.

It is reasonably well established that the evaporation of water from the insect is governed approximately by the law of saturation deficiency. Increased saturation deficiency, therefore, will cause increased loss of water from the insect. This in turn can have but two possible repercussions, either an increase of the 'input water' to the insect (to meet the increased loss), or the progressive development of desiccation in the insect. The latter event is inevitable if the former adjustment does not take place and as yet there is little evidence that it does so. Two sources of 'input water' are available in such cases as this, viz. food water and metabolic water. If either, or both, of these is to be increased, the insect must increase its rate of feeding and/or its rate of metabolism. There is little positive evidence that either process is practised by the insect, and considerable evidence to the contrary. On the positive side, Schulz (1930) according to Buxton (1932), found that meal-worms feeding on bran passed partially desiccated faeces which were 80% undigested, and in Buxton's words it thus 'appears that they eat wastefully in order to benefit from the small proportion of water rather than from the solids in the bran'. There is no indication to what extent, if any, this is a response to altered humidity conditions. With regard to increased metabolism in the larval phase in response to lowered humidity, the only example known to us is the one to which previous reference has been made of the mealworm (Buxton, 1930). On the other hand it has been shown on several occasions (Mellanby, 1932, 1934, 1935*a*, *b*; Buxton & Lewis, 1934; Gunn & Cosway, 1942) that humidity has no effect on metabolic rate. Though much of this work has been carried out on starving animals and is therefore open to the objection that the insect is not in its normal total environment, the results of Fraenkel & Blewett (1944) are not subject to this error. The work of these authors on *Ephestia*, *Tribolium* and *Dermestes* is one of the most comprehensive attempts up to the present time to examine the effect of humidity on the utilization of food by the feeding insect, and as such, is worth examination in some detail. In the course of this work assays were made of the moisture content of the food, of the amount of food eaten by the larva and the duration of the larval phase, and of the wet and dry weights of the resulting pupae. From this data we have been able to make some additional calculations which in our opinion show conclusively that in *Ephestia* and *Dermestes* (the data for *Tribolium* is incomplete), humidity has no significant effect on metabolic rate. The oxidative destruction of food material calculated in mg./diem/mg. of the average dry weight of the organism does not vary more than 1–3% over the whole humidity range employed, viz. 70 – 0% R.H. in *Ephestia* and 70 – 30% R.H. in *Dermestes*. Fraenkel & Blewett (1944) also claim that their insects do not attempt to tap the supply of food water by eating large quantities of food, though the grounds for their conclusion are somewhat obscure. The bulk of the evidence so far available suggests, therefore, that in general, insects make no attempt to counter increased evaporation either by increasing their intake of food water or by raising their metabolic rate. We can only conclude that increasing saturation deficiency automatically leads to a progressive desiccation of the insect. The work of Mellanby and Gunn & Cosway previously referred to shows that this does in fact occur in starving insects (with the one exception we have noted), and we may deduce that such is also the case in feeding *Ephestia* and *Dermestes*. It is practically impossible to determine whether

Calandra varies its rate of feeding under different moisture conditions since it is feeding entirely within the grain. Further, we have made no determinations of its rate of metabolism. We see no reason at the moment, however, to assume that its behaviour differs from that of other insects which have been the subject of investigation, and if this is so, it is impossible to avoid the conclusion that progressive desiccation results from evaporation and becomes increasingly acute with rising saturation deficiency. It is thus evident from our results that protraction of development goes hand in hand with desiccation. This may well prove to be a widespread phenomenon, for the results of other workers agree with ours in that higher S.D. causes protraction of development and we believe that the state of desiccation of the insect will prove to be dependent on the S.D. As to the explanation of this phenomenon little can be said, though the majority of facts lend support to Bělehrádek's hypothesis (1926, 1930), viz. that the rate of development and possibly other processes depend on the viscosity of protoplasm. Since the latter is dependent on the proportion of water present, desiccation of an insect is calculated to raise the viscosity of the protoplasm and thus to retard the processes of growth and development.

This view of the problem differs widely from that of Fraenkel & Blewett (1944). These workers found that at low humidities more food was eaten, developmental periods were lengthened and the final weight of the organism was smaller. They suggest that more food is required at low humidities because part of the food is utilized as water and the slower growth and smaller size are a consequence of this. In other words, part of the food is diverted from the process of growth to the production of metabolic water in order to stave off the desiccating effect of a drier atmosphere. This implies an increased rate of metabolism at the lower humidities which their data, in our opinion, as explained earlier, very clearly show does not occur. Their conclusions are based largely on the results of a control experiment on *Ephestia* designed to show that protraction of the larval phase has no effect on the 'net utilization' of food, i.e. on the ratio of the total food eaten to the amount incorporated into the body. The experiment was carried out by rearing *Ephestia* larvae at a lower temperature *but at the same* R.H. as in the main experiment. It was found that the 'net utilization' of food was unaltered and Fraenkel & Blewett conclude that since development takes considerably longer at the lower temperature the duration of the larval phase has no effect on 'net utilization'. There is, however, every reason to expect the 'net utilization' to be unaltered in this case, for the experiment is calculated merely to reduce the general rate of metabolism of the insect without in any way disturbing its water balance. Reduction of temperature under the conditions of the experiment would reduce not only the intake of food water and production of metabolic water, but also the rate of evaporation since the S.D. has been lowered. We cannot, therefore, subscribe to the conclusion that protraction of the larval phase under any circumstances has no effect on the 'net utilization' of food, for in our opinion it depends entirely on the causal factor responsible for the protraction. Fraenkel & Blewett's conclusion that the 'net utilization' is unrelated to the duration of the larval phase leads them to relate it directly to humidity. Thus, in their final analysis, humidity controls the 'net utilization' of food, and this in its turn, influences the duration of development—lower humidity by raising the 'net utilization' diverts food material from growth and thus leads to protraction of the larval phase. This interpretation we believe to be untenable for the reasons elaborated above. In our view humidity directly controls the rate of development (by influencing the water content of the insect) and it is this which regulates the 'net utilization', for protraction of the larval phase at a given temperature is likely to require greater intake of food in order to maintain the general basal metabolism for the extended period.

In conclusion, attention must be drawn to the necessarily tentative nature of many of our findings. The evidence we have brought forward in support of certain of our assumptions, however strongly presumptive, is clearly no proof that the conditions in *Calandra* are as we have suggested. The necessity for more detailed work on some species is only too clear. Determination of food utilization and respiratory rates are required in conjunction with control of temperature, atmospheric humidity, food and food water. Until such work is carried out on the feeding insect it will not be possible to draw any exact conclusions regarding the influence of environmental factors on the course of normal development.

SUMMARY

1. The effects of temperature and humidity on the duration of each instar of the life cycle of *Calandra granaria* Linn. have been examined. The insects were reared at temperatures ranging from 15 to 30° C. and at atmospheric humidities ranging from 40 to 80% R.H.

2. A method is described for assessing the effect of temperature as an independent factor.

3. The temperatures employed fall within the 'vital zone'. Extrapolation indicates the threshold temperature to be approximately 11° C. for the egg and larval instars though somewhat lower for the pupa. 30° C. is below the optimum temperature.

4. The durations of the egg and pupal stages are not affected by atmospheric humidity.

5. The duration of all larval instars is affected by moisture. It is suggested that this is largely due to atmospheric humidity and that food water is of little significance.

6. Shortage of moisture acts as an obstacle to development. Evidence is presented which indicates that drier atmospheres tend to desiccate the insect and that desiccation is responsible for retarded growth and development.

7. Since much earlier work on temperature and moisture has been done on fasting insects and, therefore, on insects deficient in one of the most important environmental factors, we suggest that our results, incomplete as they are, indicate the need for new approaches to be made. More complete data on feeding insects under controlled conditions of food, temperature and moisture are required, from which can be drawn up more complete balance sheets of development involving measurements of food utilization and respiratory rates.

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THE CHEMORECEPTORS OF THE WIREWORM (*AGRIOTES* SPP.) AND THE RELATION OF ACTIVITY TO CHEMICAL CONSTITUTION

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(With Four Text-figures)

I. INTRODUCTION

It has been shown in a previous paper that wireworms exhibit two kinds of response to chemical stimulation, biting and orientation (Thorpe, Crombie, Hill & Darrah, 1947). Biting is elicited by substances which are members of the three major food groups—carbohydrates (sugars), fats and proteins—in fairly high concentration. The orientating response is elicited by these substances at the same concentrations and by others (e.g. asparagine) at very low concentrations. It is possible by means of these responses to explain the finding and eating of food crops by wireworms in the field. In the present paper it is proposed to describe further work on the characterization of substances causing the orientation response, on food finding and on the physiology and anatomy of the chemoreceptors (cf. Moncrieff, 1944; Piéron, 1945).

Details of tests for the orientation and biting responses are described in the previous paper (Thorpe *et al.* 1947). All the work described in the present paper was carried out with *Agriotes obscurus-lineatus*. The wireworms were kept well fed before testing. The orientation tests were all performed in sand in the plate apparatus or choice chamber. Individuals used in the plate apparatus had previously been shown to respond to 2% glucose solutions. All solutions tested for this response were adjusted to *p*H 7 by HCl or NaOH. The biting tests were performed either with individual wireworms or groups of twenty as described below. The number of bites given in 24 hr. on filter papers containing the test solution was taken as the measure of palatability, and the presence of a significantly greater number of bites on these than on filter papers containing the same amount of water was taken as an indication of the activity of the solution and the sensitivity of the wireworms. The wireworms were previously tested for activity, and only individuals giving 10–50 or groups of 20 giving 150–600 bites per 24 hr. were used for the tests. All experiments were performed in darkness in a constant-temperature room; the orientation tests at 16° C. and the biting tests at 23° C. as in the previous paper (Thorpe *et al.* 1947).

II. ORIENTATION

(1) *The relation of activity to chemical constitution.* As stated in the previous paper (Thorpe *et al.* 1947) the activity of a substance is defined as minus the logarithm of the threshold concentration. That is, a substance of threshold 10^{-9} g./ml. is said to have

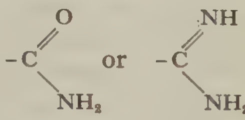
an activity of 9. In most cases tests were made at concentrations of 10^{-7}, 10^{-9}, 10^{-11} g./ml., etc., so that the activity of any substance in the table may be up to two units higher than the figure given. All the substances mentioned in this section were tested in the plate apparatus.

There is a clear distinction between those compounds which cause only orientation and those which cause both biting and orientation. In almost every case the former have high activities, 9–11, while the latter have activities of less than 3. All the biting compounds tested for orientation have proved active, the threshold being similar to that for biting. They are listed in Table 3. Several compounds of high orientation activity have been tested for biting. None elicited any response. This section is confined to compounds which cause only orientation.

An attempt was made to discover some common factor in the compounds causing orientation, as if found it would aid the selection of a bait for use in the field, and might throw light on the mechanism by which the receptor is stimulated. Forty-six compounds have been tested with this end in view. The active compounds fall into two groups.

In the first place all the amides tested are active. This is brought out in Table 1 (*a*), which shows that in the fatty acid amide series the activity due to stoichiometric amounts of amide remains roughly constant over a range extending from C_1 to C_{18}. The inactivity of two acids whose amides were shown to be active, and of ammonia itself, leaves no doubt that the amide group is responsible for the activity of these compounds.

Table 1 (*b*) shows the activity of the two common plant amides, which as will be shown later may not be entirely due to the amide linkage. The activity extends to urea and guanidine. Hence arginine is active, but when the guanidine group is removed, in ornithine, the activity disappears. Further proof that activity depends on



and not $-\overset{|}{\underset{|}{C}}-NH_2$ is given by the inactivity of glycine and alanine.

Secondly, all the other compounds found to be active are dicarboxylic acids, but the converse, that dicarboxylic acids are active by no means applies. The activities of ten dicarboxylic acids are given in Table 2 (*a*). There is no simple relationship between activity and chain length.

Of the remaining active compounds aspartic acid is a derivative of succinic acid. The view that the activity is due to the dicarboxylic acid skeleton is reinforced by the fact that the activity of a derivative in which the amino group is blocked is not significantly reduced. On the other hand, glutamic acid, which has been tested several times in a highly purified state, is very active though derived from the inactive glutaric acid in the same way as aspartic from succinic. Malic acid again is derived from succinic by the substitution of one hydroxyl group, but the introduction of a second hydroxyl produces the inactive tartaric acid. Citric acid is one of the few compounds known to possess an activity between 3 and 9, and can be considered as a substituted succinic or glutaric acid. Because the activity of ethyl succinate is higher than that of succinic acid, it would seem likely that the activity of the ester is not due to the slight hydrolysis which occurs in aqueous solution.

Table 1. *Amides tested for orientation*

(*a*) Amides of fatty acids

	Activity	Activity + log mol. wt
Formamide	11	12·5
Acetamide	9*	10·8
Propionamide	9	10·9
Caprylamide	9	11·2
Stearamide	8	10·5
Acetic acid	None	
Sodium acetate	None	
Sodium propionate	None	
Ammonia	None	

(*b*) Other amides and urea derivatives

	Activity
Asparagine	9
Chloracetyl asparagine	9
Glutamine	9
Succindiamide	9
Urea	9
Urethane	11
Guanidine	9
Arginine	9
Ornithine	None
Glycine	None
Alanine	None

* < 10.

Table 2. *Dicarboxylic acids tested for orientation*

(*a*) Saturated dicarboxylic acids

	Activity
Oxalic	None
Malonic	None
Succinic	9
Glutaric	None
Adipic	9
Pimelic	9
Suberic	None
Azelaic	None
Sebacic	4
Hexadecane 1–16 dicarboxylic	9

(*b*) Substituted dicarboxylic acids, etc.

	Activity
Aspartic	11
Chloracetyl aspartic	9
Malic	9
Tartaric	None
Citric	5
Glutamic	9, 11
Fumaric	None
Ethyl succinate	11

In view of the small number of compounds tested the two groupings of active substances should be treated with reserve. The activity of the amide group seems definite. That many dicarboxylic acids are active is also true, but no rule is apparent for predicting if a given dicarboxylic acid will be active or not. The subject of chemical constitution and taste and odour has recently been extensively discussed by Moncrieff (1944).

Several of the active compounds are found in plants. In particular, the amino-acid amides, asparagine and glutamine are of universal occurrence, reaching high concentrations in certain seedlings as secondary products of the breakdown of the seed protein. Arginine also is found in considerable amounts in some seedlings, and is widely distributed at lower concentrations in the mature plant.

A small number of experiments were performed to discover whether active substances are inhibited by other compounds. The activity of asparagine remained unaltered when solutions of this substance were adulterated with 1 % malonic acid. Sodium chloride in 2 and 4% solutions was likewise without effect on the activity of asparagine. But 2% glucose solutions were rendered inactive by the addition of 2% sodium chloride although not by 1% solutions. The last result agrees with the inhibiting of biting of glucose by sodium chloride (Table 6).

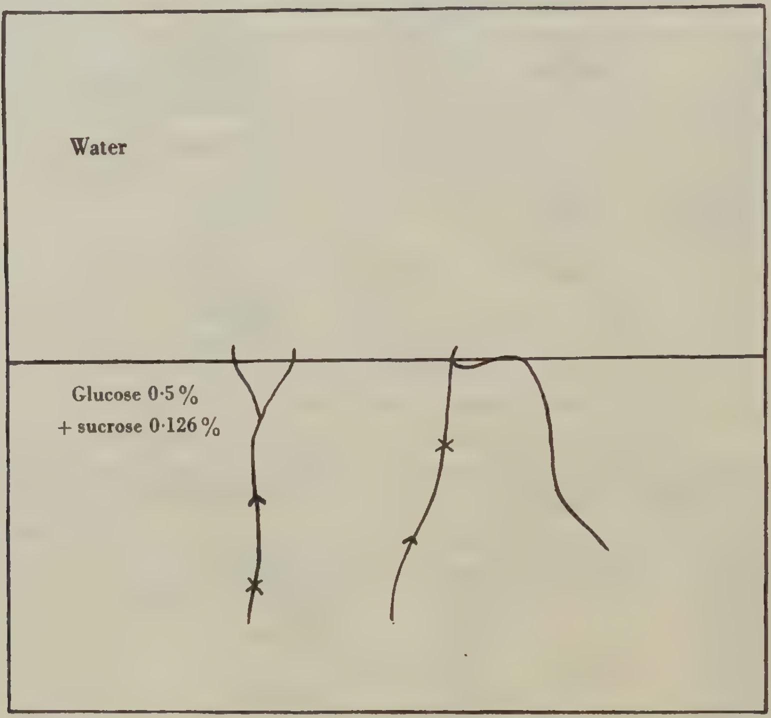


Fig. 1. Tracks made by wireworms showing the response to mixtures of subthreshold concentrations of glucose (0·5 %) and sucrose (0·126 %) solutions. The arrows indicate the position and direction in which the wireworms started, the crosses where they were found after 2 hr. The two substances summate.

(2) *Summation of substances causing orientation.* Because of the extremely low concentrations in which they are active it is not possible to test the substances shown in Tables 1 and 2 for summation. This could, however, be studied with substances whose activity is approximately 2, i.e. those which also cause biting. Only two substances were tested, viz. glucose and sucrose. With fed wireworms no orientation is caused by 0·5% solutions of glucose or 0·126% solutions of sucrose, while 0·8% solutions of glucose and 0·25% solutions of sucrose are active. The activities of these sugars are therefore: glucose 2·1 and sucrose 2·6. Mixed solutions containing 0·5% glucose and 0·126% sucrose are active, showing that summation has occurred (Fig. 1).

(3) *Competition of substances causing orientation.* It has already been shown (Thorpe *et al.* 1947, § IV (iv)) that wireworms respond to a gradient of concentration of both glucose and asparagine. Competition between different substances causing orientation was tested in the choice chamber by impregnating the sand on one side with a solution of one substance and that on the other side with the same volume of a solution of the other substance. The results, with substances at the concentrations shown, are given in Table 4.

Table 3. *Miscellaneous substances tested for orientation*

(*a*) *Substances of activity less than 3 which also cause biting**

	Activity
Glucose	2·1
Fructose	2
Sucrose	2·6
Triolein	2
Peptone (B.D.H. and 'Bacto')	2·1
Tannin	2

(*b*) *Inactive substances not previously tabulated*

- Acetaldehyde
- Ethyl acetate
- Alcohol
- Acetone
- Aniline

* These are the only compounds causing biting which have been tested for orientation.

Table 4. *The competition of substances causing orientation*

Substances opposed in choice chamber		Wireworms counted on each side	
(*a*)	(*b*)	(*a*)	(*b*)
Asparagine 10^{-9}	Glucose 8×10^{-3} (0·8 %)	150	157 = 51 %*
Asparagine 10^{-9}	Glucose $1·26 \times 10^{-2}$ (1·26 %)	65	124 = 65·6 %
Asparagine 10^{-7}	Asparagine 10^{-7} + glucose $1·26 \times 10^{-2}$ (1·26 %)	140	297 = 68 %
Asparagine 10^{-7}	Asparagine 10^{-7} + sucrose 2×10^{-2}	222	407 = 64·7 %
Asparagine 10^{-9}	Peptone 2×10^{-2} (2 %)	64	116 = 64·4 %
Asparagine 10^{-7}	Asparagine 10^{-7} + peptone 2×10^{-2}	271	405 = 60 %

* Standard error 4·5 % (see Thorpe *et al.* 1947).

When threshold concentrations of asparagine and glucose (0·8 % corresponds to activity 2·1) are opposed, no orientation occurs in either substance. But higher concentrations of glucose and of peptone cause orientation in spite of the competition of asparagine at threshold concentration. When asparagine at low concentration is opposed to mixtures of asparagine at the same concentration with either glucose, sucrose or peptone at high concentration, the wireworms collect in the mixtures. All these results are in keeping with the fact that wireworms respond to a gradient of concentration, but here the steps in the gradient are made by different substances.

(4) *Root secretions and food finding.* It has already been shown that wireworms will orientate round the roots of food plants growing in sand (Thorpe *et al.* 1947, § III). It is therefore of interest to discover whether active substances come out of the roots of growing plants into the surrounding soil (or sand).

Potatoes were grown under glass in sand and in garden soil in washed pots of about 1 l. Small amounts of water were added every other day to make up for evaporation. Leaching by excessive watering was avoided. After 5 weeks the potatoes in soil being about 9 in. high and those in sand half as big, water was passed through each pot until ½ l. had drained through. This extract was passed through a second time. A control of soil without potatoes was extracted in the same way after 2 weeks.

The control was inactive, while the extract from soil in which potatoes had grown had an activity of 4 and that from sand 6. The sand extract was equivalent to 1 part of aspartic acid in 10^5 of water. It gave a deep red ninhydrin colour sufficient to overcome the blue colour which aspartic acid would have given at that concentration. That the extract from sand was more active than that from soil is accounted for by greater adsorption on the soil particles.

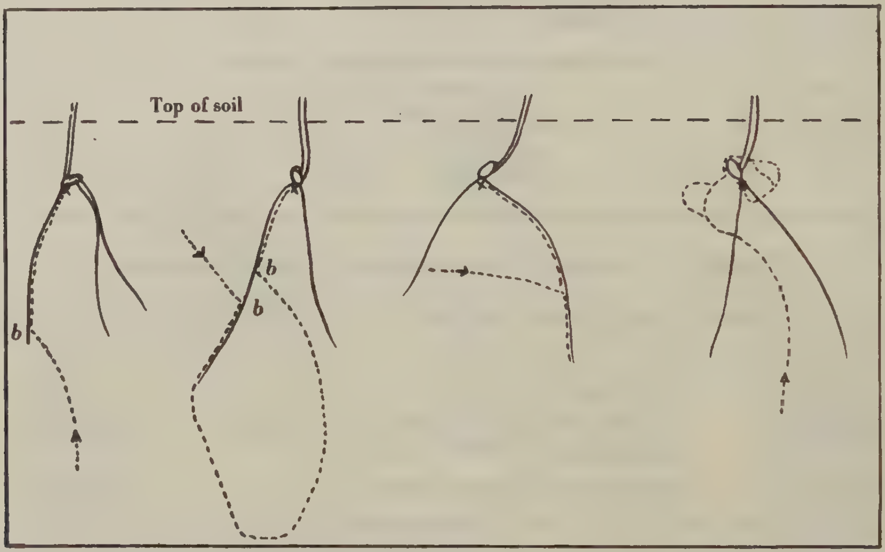


Fig. 2. Tracks made by wireworms in the region of germinating wheat. The arrows represent the position and direction in which individuals started, the crosses where they finished and began feeding on wheat grain. The letter *b* indicates points at which the root was bitten.

This experiment shows that active compounds are excreted in small amounts by the growing potato plant. Since any plant product will be destroyed by soil there will be a concentration gradient from either individual roots or the root system as a whole. As the activity of the extract made is at least 4, even in the case of soil, part of this gradient must be within the range of concentration within which substances can be detected by wireworms.

Experiments were now carried out to observe the behaviour of wireworms in the vicinity of growing plant roots. Wheat grains were allowed to germinate individually in fen soil between glass plates, and when a sufficient root had been put out a single wireworm was placed near each grain and its behaviour observed. Examples of the tracks made are shown in Fig. 2. The arrow indicates the position and direction in which a wireworm started, the cross where it finished after 2 or more hours. A wireworm will wander at random through the soil until it strikes a root. It may then either bite it and follow it in either direction, or follow it without previous biting. On reaching the distal end of the root it may either pass beyond it out into the soil, or reverse along its track. In this way it may be led to the grain. Wireworms also show a tendency to make tracks round the grain even where there are no roots. On reaching the grain a wireworm will usually remain there feeding for several hours or days.

Wireworms clearly tend to be retained in the vicinity of the roots and grain, and it may be presumed that this is due to the active secretions shown to be given off by the potato. By sloughing off the cortex behind the root hairs, roots probably make holes larger than the root itself and so make an easy passage, and root hairs are damaged or pushed off as wireworms push along beside the root. It is known that they bite at random in the absence of active biting substances, for they will bite filter paper impregnated with water only (Thorpe *et al.* 1947). This would account for the initial biting of roots and grain. Active biting substances (e.g. sugars) are present in most plant juices in active concentrations, and these, released by the initial wound, would continue to elicit the biting response. The root system thus provides an extended trap along which wireworms may be led to the grain or tuber in the centre, where they remain to feed.

III. BITING

(1) *Summation of substances causing biting*. The number of bites elicited by glucose, sucrose, peptone, triolein and tannin, respectively, per 20 wireworms in 24 hr., is shown in Table 5. Except with triolein 5 drops of each solution (0·25 ml.) were placed on each filter paper (5 cm. diameter). In tests with triolein filter papers were impregnated with the amounts shown. The data for glucose have already been published (Thorpe *et al.* 1947, Fig. 7). With all the substances, except tannin, as the concentration decreased the rate of biting fell from a maximum of approximately 350 bites per 20 wireworms in 24 hr. to a value not significantly different from that on the control filter papers containing water. With all substances the number of bites on the latter varied between 0 and 50 per 20 wireworms in 24 hr. With tannin a similar decrease in biting occurs, but the maximum is much lower, viz. approximately 130 per 20 wireworms in 24 hr.

Summation was tested as follows. Mixtures were made containing two substances each at half the lowest concentration which caused maximum biting. The rate of biting caused by such concentrations of each substance alone was always very far below the maximum, as shown in Table 5. But with such mixtures of glucose + sucrose, glucose + peptone, glucose + triolein and peptone + triolein the rate of biting was again at the maximum. These substances therefore summate, each being equivalent to the other in stimulating the chemoreceptors. With the mixture of glucose + tannin the rate of biting was up to the maximum for tannin but well below that for glucose. Some form of summation has therefore occurred and it may be concluded that these two substances also affect the same receptors. The number of bites on the water controls was 0–50 as before.

(2) *Substances inhibiting biting*. It was shown in the previous paper (Thorpe *et al.* 1947, Fig. 6) that the rate at which 2 % glucose is bitten is greatest with *p*H 6–8, and decreases in more acid or alkaline solutions. Biting of 2 % solutions of glucose is also inhibited by a number of substances, viz. lead acetate, quinine, allylisothiocyanate and common salt (Table 6). Five tests were performed with each substance with fed wireworms. The rate of biting was reduced in proportion to the concentration of the inhibitor (see Table 5, and Thorpe *et al.* 1947, Fig. 7). Other substances which do not themselves elicit biting, viz. saturated β-pentacetyl glucose (1 part in 1175 parts of water at 18·5° C.) and 2 % acetamide, did not inhibit this response.

Table 5. *The summation of different substances eliciting the biting response*

Substance	No. exps.	No. bites per 20 wireworms in 24 hr.	
		Average	Range of variation
Glucose 2·0 %	12	342	150–550
1·26 %	30	355	150–600
0·63 %	10	81	30–180
0·5 %	10	28	5– 50
Sucrose 1·0 %	5	337	180–550
0·5 %	10	329	200–550
0·25 %	10	89	10–190
0·126 %	5	34	10– 50
Peptone 2·7 %	5	397	310–450
1·8 %	10	377	300–460
0·9 %	10	61	10–170
0·5 %	5	22	0– 40
Triolein 62 mgm.	5	340	280–400
17 mgm.	10	333	300–400
10 mgm.	10	46	20– 80
Gallotannin (B.D.H.)			
5 %	5	139	55–220
1 %	10	123	50–200
0·5 %	10	13	5–100
Glucose 0·63 % + Sucrose 0·25 %	10	293	140–360
Glucose 0·63 % + Peptone 0·9 %	10	300	200–430
Glucose 0·63 % + Triolein 10 mgm.	10	426	230–700
Peptone 0·9 % + Triolein 10 mgm.	5	515	350–800
Glucose 0·63 % + Gallotannin (B.D.H.) 0·5 %	10	170	50–400

In all experiments the number of bites on the water controls varied between 0 and 50 per 20 wireworms in 24 hr.

With the evidence available it is impossible to decide whether the four inhibitory substances affect biting through upsetting metabolism or through the sense organs. It is probable that they are toxic at concentrations at which they take effect. Tattersfield & Roberts (1920) showed that allyl-iso-thiocyanate vapour is a contact poison. But as wireworms tested with these four substances seem unaffected, it is possible that their behaviour towards them is under sensory control and that they stimulate either the chemoreceptors proper or the common chemical sense. In the latter case they would be classed as irritants. Woodworth (1938) has shown that the wireworm *Pheletes* (*Limonius*) *canus* has a closing mechanism of hairs in the buccal cavity which prevents the passage of arsenical poisons in solution into the alimentary canal. This mechanism is under sensory control and dyes and other non-poisonous substances, as well as food, are taken in, showing that discrimination is exercised. Possibly the biting behaviour of *Agriotes* larvae when presented with solutions of 2% glucose mixed with inhibitory substances is of the same kind (cf. Frings, 1945, 1946).

Table 6. *The inhibition of biting 2% glucose by various substances (5 tests with each substance)*

Substance	No. of bites per 20 wireworms in 24 hr.	
	Average	Range of variation
Allyl-iso-thiocyanate 0·01%	320	200–450
0·1%	115	60–170
Lead acetate 0·1%	345	200–400
2%	80	20–120
Quinine 0·01%	310	220–370
0·1%	80	30–110
NaCl 1%	300	250–350
2%	30	0– 45
β-pentacetyl glucose saturated	310	270–340
Acetamide 2%	260	210–370

In all experiments the number of bites on the water controls varied between 0 and 50 per 20 wireworms in 24 hr.

IV. LOCATION AND MORPHOLOGY OF THE CHEMORECEPTORS

(1) *Location of the chemoreceptors.* Lees (1943) points out that wireworms have five types of sensillae on the head appendages: (*a*) Campaniform organs are distributed on the dorsal surfaces of the antennae, galeae, ligula and maxillary and labial palps, with a few on the more basal regions of the mouth parts. (*b*) Peg organs are found on the labial and maxillary palps and galeae, mostly on the ventral surfaces. They are absent from the antennae. (*c*) Thick walled bristles are found on all the head appendages. (*d*) Minute projections are borne on the end of the distal segments of the labial and maxillary palps. (*e*) There is a cup-shaped structure on the distal segment of the antenna.

In order to locate the chemoreceptors, appendages were removed, in various combinations, in batches of 10 wireworms each, by inserting a finely pointed needle through the basal intersegmental membrane. The wireworms were held with the finger without anaesthetic on a piece of damp filter paper and the operation performed under a binocular microscope. All organs were removed bilaterally. After a period of a week for recovery, they were tested for both orientation and biting (cf. Crombie, 1941; Frings, 1941, 1944).

Wireworms continued to orientate in solutions of 2% glucose and 10^{-3} and 10^{-9} asparagine when the following combinations of organs were removed: (*a*) antennae, (*b*) antennae, labial palps and ligula, (*c*) antennae, maxillary palps and galeae, (*d*) antennae, labial palps + ligula and maxillary palps (leaving galeae), (*e*) labial palps and ligula, maxillary palps + galeae (leaving antennae). When antennae, labial palps + ligula and maxillary palps + galeae were all removed, no orientating response was observed with any substance. Wireworms will thus orientate when either the antennae or the galeae are present (Fig. 3). The chemoreceptors concerned must therefore be on these appendages and the minute projections on the ends of the labial and maxillary palps are not necessary for this response. Campaniform organs and thick walled bristles are common to both the galeae and antennae. It seems unlikely that the former are chemoreceptors since Pringle (1938*a*, *b*) has shown that organs of this type in other insects function as proprioceptors and show no response to chemical stimuli. The thick walled bristles are also unlikely to be chemoreceptors (of which the presence of thin or perforated walls is usually considered a necessary feature: Wigglesworth, 1939, pp. 149 et seq.); they are more probably tactile in

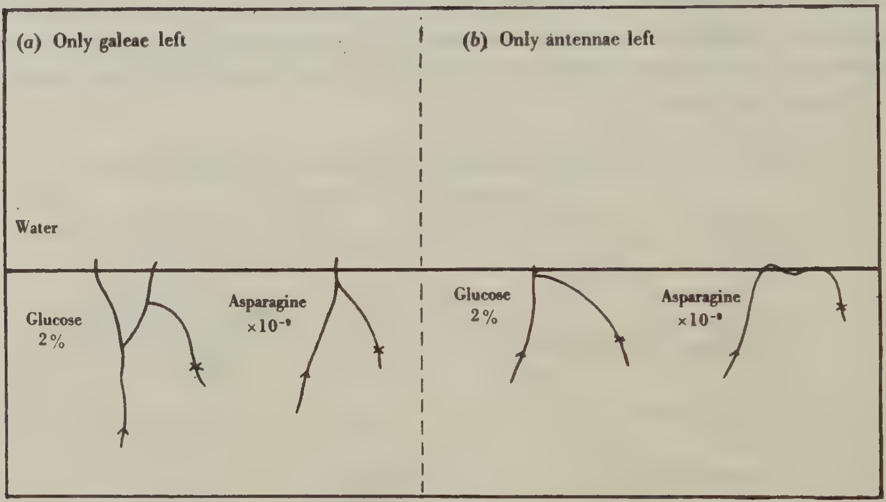


Fig. 3. Tracks made by amputated wireworms in response to 2% glucose and 10^{-9} asparagine solutions, respectively. (*a*) antennae, labial palps + ligula, and maxillary palps removed, leaving galeae; (*b*) labial palps + ligula, maxillary palps + galeae removed, leaving antennae. The arrows indicate the position and direction in which the wireworms started, the crosses where they were found after 2 hr.

function. If these conclusions are correct it would seem that the peg organs on the galeae and other mouth parts and the cup-shaped structure on the antennae must be the chemoreceptors.

The results of biting tests with wireworms from which various appendages had been amputated are shown in Table 7. When any appendage bearing peg organs is present the normal biting response is elicited. With all peg organs removed and only antennae present the number of bites is not significantly different from that on the control filter papers containing water. The antennae therefore do not carry receptors whose stimulation leads to this response. Normal biting occurs when only the galeae are present, showing that the minute projections on the tips of the labial and maxillary palps are not necessary for this response.

Table 7. *The biting response of amputated wireworms elicited by* 2% *glucose*

Appendages removed	No. wireworms tested	No. of bites per wireworm in 24 hr.	
		Average	Range
No appendages removed	30	24	10–50
Antennae	10	22	10–50
Antennae, labial palps + ligula	5	31	15–50
Antennae, maxillary palps + galeae	5	25	0–90
Antennae, labial palps + ligula, maxillary palps (leaving galeae)	10	22	10–40
Labial palps + ligula, maxillary palps + galeae (leaving antennae)	10	1	0– 2
Control filter papers with water	30	1·1	0– 5

If it is true that the campaniform organs and the thick walled bristles are not chemoreceptors, the following picture thus presents itself. The peg organs are sensitive to 2% glucose solution and 10^{-3} and 10^{-9} asparagine solutions. Their stimulation by any of these solutions elicits orientation, while their stimulation by glucose solutions elicits biting. The cup-shaped sensillae on the antennae are also sensitive to 2% glucose solutions and 10^{-3} and 10^{-9} asparagine solutions, but their stimulation elicits only orientation.

(2) *Morphology of the supposed chemoreceptors.* The external features of the wireworm are described in detail by Roberts (1921), Guéniart (1934) and Subklew (1934), the first-mentioned author describing the mouth parts in detail. The morphology of the sensillae on the mouth parts and antennae were examined by cutting sections of freshly moulted specimens. After fixing in Carnoy the appropriate appendages were embedded by the methyl benzoate and celloidin method and sections of 3–10 μ thickness cut and stained with either iron haematoxylin or with Mallory's phosphotungstic acid iron haematoxylin. Fig. 4*a*, *b* represent the two organs which the evidence presented in the previous section suggests to be the chemoreceptors. Fig. 4*b* and *c* (a campaniform organ) are drawn from sections prepared by Dr A. D. Lees and stained with iron haematoxylin. Fig. 4*a* is drawn from a section stained with Mallory's stain.

The cup-shaped organ or multi-celled basiconic sensilla (Snodgrass, 1935) on the distal joint of the antenna, shown in Fig. 4*a*, has at its base a ring of cuticle. The cup or dome itself has a thick wall which appears to be perforated by fine canals which at first run in parallel lines from the outer surface inwards, then ramify and open on the irregular inner surface of the dome. A number of nerves run down to join sense cells in the distal and proximal segments of the antenna. A similar organ was found on the antenna of the larva of the beetle, *Rhizopertha dominica* (Crombie, 1944). Stain is taken up by the unsclerotized intersegmental regions of the cuticle, which Lees (1943) believes to be one of the main sites of evaporation in unsaturated air, while the sclerotized regions remain unstained.

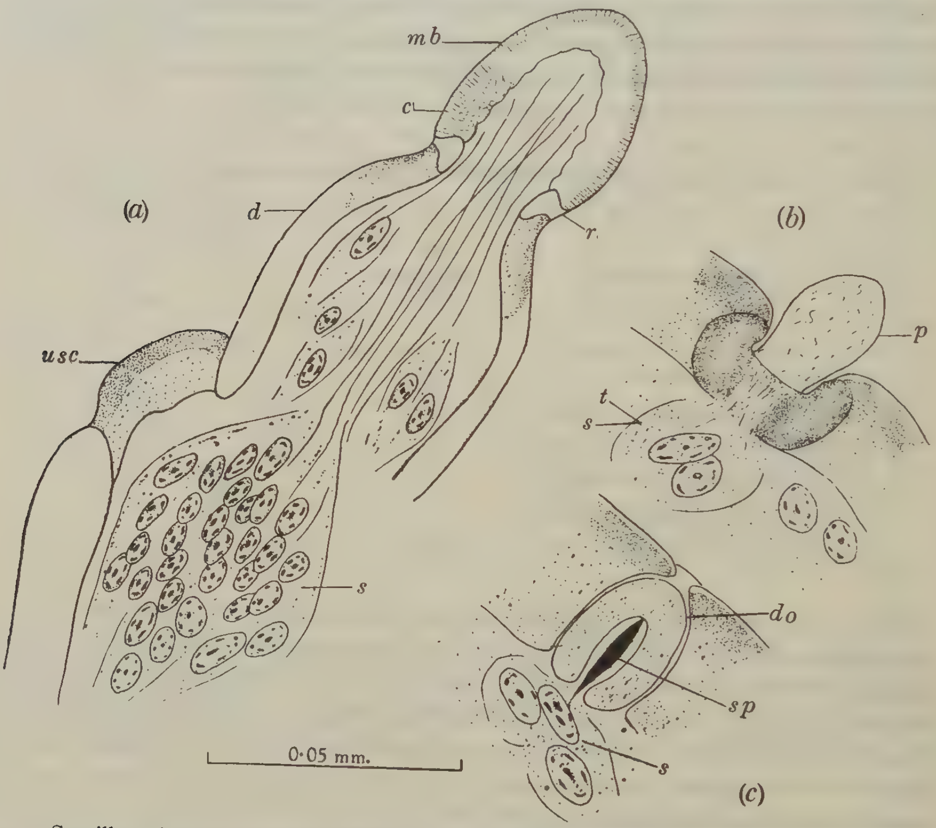


Fig. 4. Sensillae of *Agriotes lineatus-obscurus*. (*a*) longitudinal section of antenna with multiple-celled basiconic sensilla; (*b*) peg organ (thin walled sensilla) on maxillary palp; (*c*) campaniform sensilla on maxillary palp.

c = canals in dome of multiple-celled basiconic sensilla; *d* = distal joint of antenna; *do* = dome of campaniform sensilla; *mb* = multiple-celled basiconic sensilla; *p* = peg organ or thin walled sensilla; *r* = cuticular ring; *s* = sense cells; *sp* = scolopale; *t* = trichogen cells; *usc* = unsclerotized part of cuticle.

The peg organs, of which one is shown in Fig. 4*b*, are each set in a cup. The walls of the sensilla are very thin, and its contents faintly stained, suggesting that it contains fluid during life (cf. Crombie, 1944). Immediately beneath the cup lie what are probably trichogen and sense cells.

The campaniform sensilla shown in Fig. 4*c* has the parts characteristic of these organs. A 'sense rod' or 'scolopale' communicates with the sense cells below, and is itself inserted into a cuticular dome which is embedded in the cuticular wall of the insect (Wigglesworth, 1939, p. 133).

V. DISCUSSION

In the study of animal behaviour, while no conclusions can be drawn about an animal's powers of discrimination if two or more stimuli elicit the same response, because the same behaviour may be elicited by diverse stimulation of the receptors, if the animal behaves differently towards two stimuli it follows that they must affect the receptors differently. Furthermore, if two stimuli summate then one is equivalent to the other in stimulating the receptors, which suggests that both stimulate the same receptors.

On these principles it is clear that the wireworm's receptors are stimulated differently by substances such as glucose which elicit both biting and orientation and by substances such as asparagine which elicit only orientation. Without implying anything beyond the observed facts of behaviour, it may thus be said that the wireworm's receptor system enables it to distinguish between these two groups of substances. That the two responses concerned are distinct is shown by the observation that according to their physiological state wireworms may exhibit either without the other (Thorpe *et al.* 1947, § IV, (iii)). The substances eliciting biting must also be distinguished from those which inhibit this response, if the latter do not merely upset metabolism. But the causes of the inhibition of both biting and orientation in glucose by sodium chloride and other substances are unknown. Summation occurred between all the pairs of bitten substances tested, suggesting that they all stimulate the same receptors. The evidence points to these being the peg organs borne on the mouth parts. Summation also occurs with glucose and sucrose when tested for orientation. It seems probable that both the peg organs and the cup-shaped sensillae on the antennae are the receptors concerned in eliciting this response. It appears therefore that stimulation of the peg organs by glucose will cause both orientation and biting, and their stimulation by asparagine orientation. Further evidence that both biting and orientation in glucose result from the excitation of the same receptor is provided by the observation that when wireworms are starved the thresholds of both responses to this substance descend together (Thorpe *et al.* 1947, § IV (ii*b*) and Fig. 7). Stimulation of the antennal sensillae by glucose or asparagine causes orientation only. These therefore appear to be additional 'distance receptors'.

The work of von Frisch (1934) has shown that some insects, e.g. the honey-bee, can distinguish between different substances which are respectively sweet, bitter, salt and sour to man. It is interesting to compare the effects of different substances on the biting response of wireworms with the sensations of taste which they produce in man. Some substances which are sweet to us (sugars) and some bitter substances (tannin) elicit the biting response in wireworms, while other sweet (lead acetate) and other bitter (quinine) substances inhibit this response. Again, other sweet substances (saccharine, glycerol) and other bitter substances (β-pentacetyl glucose) are inactive. Salt and sour substances inhibit biting. Thus different substances causing the same sensation in man are distinguished by the wireworm, and different substances causing the same behaviour in the wireworm are distinguished by man (see Moncrieff, 1944).

VI. SUMMARY

1. This paper continues the work on the responses of wireworms (*Agriotes* spp.) to chemical stimulation, published in a previous paper (Thorpe *et al.* 1947), by a further analysis of the relation of activity to chemical constitution and of food finding, and the investigation of the anatomy and physiology of the chemoreceptors.

2. Substances present in food plants elicit two responses in wireworms, orientation and biting. There is a clear distinction between those compounds which cause only orientation and those which cause both biting and orientation. In almost every case the former have high activities (9–11) while the latter have activities of less than 3. All the biting compounds tested for orientation have proved active, the threshold being similar to that for biting.

3. Substances causing orientation fall into two classes, viz. amides and urea derivatives, and dicarboxylic acids. In view of the small number of compounds tested the two classes of active substances should be treated with reserve. In the first, it seems definite that the amide group is responsible for activity. But while all the other compounds found to be active fall into the second class, i.e. are dicarboxylic acids, it is by no means true that all dicarboxylic acids are active and no rule is apparent for predicting whether a given dicarboxylic acid will be active or not.

4. Substances causing both orientation and biting summate when either response is tested. The following pairs of substances summated when tested for the responses indicated: glucose and sucrose for orientation and biting; glucose and peptone, glucose and triolein, peptone and triolein and glucose and tannin for biting.

5. Orientation occurs in a gradient of concentration of which the steps are formed by different substances, as well as when the steps are formed by one substance as was shown in the previous paper.

6. Active substances are secreted in small amounts by the roots of growing potato plants. These would explain the orientation of wireworms to the roots of growing plants (e.g. wheat). It is thus likely that the root system forms an extended trap along which wireworms may be led to the grain or tuber in the centre, where a wound caused by their random biting would release active compounds in sufficient concentration for them to remain and feed.

7. The biting of glucose is inhibited by a number of substances, viz. lead acetate, quinine, allyl-iso-thiocyanate and common salt, as well as by acid and alkaline solutions. Common salt also inhibits orientation in glucose, but not in asparagine.

8. The chemoreceptors which appear to be involved in these responses are of two kinds: peg organs, located on the labial and maxillary palps and galeae, whose stimulation leads to both orientation and biting; and a cup-shaped sensilla on the

distal segment of each antenna, whose stimulation leads to orientation only. Both asparagine and glucose stimulate both kinds of receptor.

We wish to thank Dr A. D. Lees for the loan of the sections from which the sensillae in Fig. 4*b* and *c* are drawn. This paper concludes the publication of the work carried out with our colleagues, Drs W. H. Thorpe and R. Hill, F.R.S.

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THE REACTIONS OF *PYGOSTEUS PUNGITIUS* L. TO TOXIC SOLUTIONS

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(With Eight Text-figures)

1. INTRODUCTION

The gradient tank method of experimentation was devised by Shelford & Allee (1913), who used it to observe the reactions of fish to dissolved oxygen and carbon dioxide. A number of other studies on the same lines followed; Wells (1913, 1915 *a*, *b*) also dealt with oxygen and carbon dioxide, with gradients of acidity and alkalinity, and with the chlorides, nitrates and sulphates of ammonium, calcium and magnesium. Later the work extended to pollutant substances of a toxic nature; Shelford (1917, 1918) studied the reactions of several species of fish to the various waste products associated with the manufacture of coal-gas. This study included ammonia and ammonium salts, aniline, hydrogen sulphide, sulphur dioxide, carbon bisulphide, acetone, phenol, cresols, naphthalene, carbon monoxide and tar acids; and Wells (1918) contributed a further study with carbon dioxide and carbon monoxide. A description of the apparatus used is included in Shelford's book on *Laboratory and Field Ecology* (1929, p. 85).

These investigators claimed that fish recognize and avoid certain substances, such as carbon dioxide, quinoline, high concentrations of sulphur dioxide and water of high acidity, but that they fail to detect many substances of high toxicity, swim into the solution, become 'intoxicated' and thereafter avoid the water end of the tank. In a long list of 'positive' substances they include ammonia, carbon monoxide, paracresol, phenol, xylene, acetone and pyridine.

The method of study is open to some measure of criticism. The use of a *gradient* seems advisable when the fish is required to select the optimum concentration of a substance, but in dealing with substances of high toxicity, of which extremely low concentrations may be ultimately fatal, it is preferable to employ an arrangement in which water and solution are sharply differentiated so that the fish is given every opportunity of discriminating between water and a definite concentration of the substance tested. Furthermore, the time during which the movements of the fish were recorded was generally limited to 10 or 12 min., and often bears no relation whatever to the degree of toxicity of the solution employed; thus Wells (1918) describes an experiment to determine the reactions of the black bullhead (*Ameiurus melas*) to carbon monoxide; the chart records the movements of the fish for 12 min. only, the concentration employed is 0·5 c.c. CO/l., but according to his table (p. 562) an 11·7 c.c./l. solution is only fatal in 9 hr. 55 min. Nevertheless, the fact that the fish spent most of its time at the solution end of the tank is taken as evidence that fish are 'positive' to carbon monoxide. Although many control experiments were run, with water at both ends of the tank, these control experiments do not seem to have been conducted with the particular fish whose reactions to the solutions were recorded. The control experiments of the writer, and those of Shelford (1917, chart 1), show that the behaviour of individual fish under control conditions may vary considerably.

Lastly, the gradient tank apparatus seems to have been somewhat unsatisfactory. Wells (1915*b*, p. 246), in his experiments with salts, found that with the solution running at the standard rate a sharp vertical gradient is also produced; at one end of the tank the solution appears to have been six times more concentrated at the bottom than at the top. Consequently it is extremely difficult to assess the concentration differences the fish encounters in its movements to and fro.

The writer (1935, 1938, 1939, 1940*a*, 1947) has investigated the degree of toxicity of heavy metal salts to fish and the nature of their lethal action, but no attempts appear to have been made to ascertain whether fish can recognize and avoid these substances. The present study deals with the reactions of the ten-spined stickleback to mercuric chloride, zinc sulphate and copper sulphate, and for comparative purposes a number of experiments were run with chloroform, alcohol and formalin. The apparatus provides a sharp differentiation between water and a known concentration of the toxic solution, enables the reactions of the fish to be checked by rapid reversals of the direction of flow of water and solution, the periods of observation are considerably lengthened, and in every experiment water was run in at both ends of the apparatus for the first 10 or 15 min. so that control observations were made on all the fish used. *Pygosteus pungitius* was found very suitable material as it is plentiful locally, and its small size obviates the necessity for cumbersome apparatus and the preparation of large volumes of solutions.

2. APPARATUS AND METHOD

The general scheme of the apparatus employed is shown in Fig. 1, which is largely self-explanatory. Aspirators *A* and *C* are filled with tap water, *B* and *D* with the solution to be tested; solution and water are fully aerated. The 'gradient tank' consists of a horizontal glass tube, 30 mm. internal diameter, 58 cm. long, capacity about 400 ml. The apparatus is disconnected at one end, the tube opened and the fish introduced. Then the apparatus is reassembled and by temporarily tilting the tube and opening clips 3 and 4 it is completely filled with water. When all air is expelled clip 3 is closed. The outlet tubes are also filled and a sufficient quantity of water introduced into the bubble-traps *E* and *F*.

Sufficient time is now allowed for the fish to become accustomed to their new surroundings. If they are gently handled and not subjected to any great temperature change they become quiet in 10–15 min. and either rest perfectly still on the bottom or swim lazily up and down. Then clips 1 and 4 are opened and screw-clip 6 is released so that water flows in at both ends and out at the outlets *G*. The rate of discharge at *H* is regulated to about 300 ml./min.

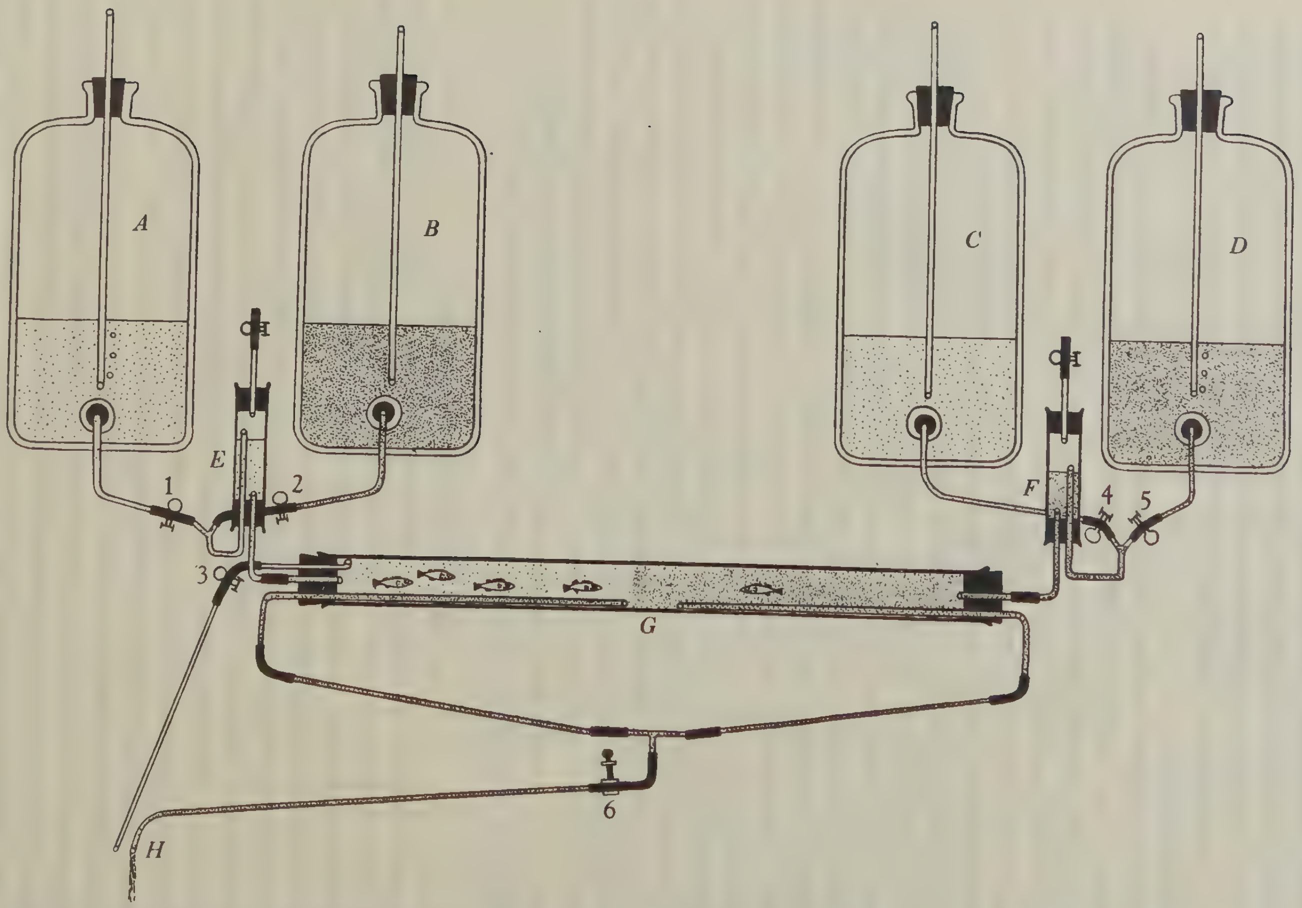

Fig. 1. General scheme of apparatus. *A*, *B*, *C*, *D*, 10 l. aspirators; *E*, *F*, bubble traps; 1, 2, 3, 4, 5, pinch clips; 6, screw clip; bungs and rubber connexions black.

The stop-clock is started, and every half minute or minute, according to the duration of the experiment, the approximate positions of the fish in the tube are recorded. Two parallel lines on graph paper represent the length of the 'gradient tank', and minutes are marked off on a vertical scale. Water is run in at both ends for 10 or 15 min. The behaviour of the fish under these conditions varies somewhat; all may rest perfectly still at various points on the bottom, or they may swim up and down and eventually show a preference for one end. The reason for this preference could not be discovered, as in one experiment the fish might congregate on the left side, while in another experiment conducted immediately afterwards with identical lighting conditions, etc., they might show an equal liking for the right. A uniform white background was provided, and the only illumination was a single 75 W. light suspended $3\frac{1}{2}$ ft. above the middle of the tube. It was soon discovered that the fish showed little reaction to external lights and objects but reacted sharply to bubbles or other objects within the tube, and to vibrations of the experiment bench. Hence such disturbances were carefully avoided.

After 10 or 15 min., by opening and closing the appropriate clips, the water flow is stopped on one side and solution run in instead. The traps *E* and *F* prevent the entry of bubbles and serve to render the replacement of water by solution less sudden. The positions of the fish are recorded periodically, and notes made on their behaviour. In general, the solution is run in on the side for which the fish show a preference.

With a flow of 300 ml./min. at *H* an extremely sharp differentiation is obtained, one-half of the tube being filled with water, the other half with solution. This can be checked with dyed solution, when the distance between the two outlets (*G*) can be adjusted for best results. Energetic swimming causes temporary mixing in the middle of the tube, but the sharp separation of water and solution is almost immediately restored. The use of dyes also shows that a complete reversal of water and solution can be effected in 3–4 min. with the standard rate of flow. A little difficulty was encountered with concentrated solutions (e.g. $N/10$ $CuSO_4$) whose high specific gravity tended to make them spread along the water half of the tube, but here again experiments with dyes showed that this could be overcome by running water and solution at a slightly increased rate. No dye was used, of course, in the experiments in which the movements of the fish were recorded.

The fish used were obtained from a local ditch and kept in the laboratory for 2–7 days before use; 25–28 mm. was found the most suitable size, though 30 mm. fish could turn round in the tube with no difficulty. They usually showed no reaction to the current, though when much enfeebled by the toxic effect of the substances studied they tended to float on the current towards the outlets. The experiment was usually discontinued at this stage.

The room temperature was maintained at 15° C. Solutions and tap water were fully aerated and allowed to stand 3–4 hr. before the experiment was begun in order to ensure equality of temperature. At temperatures above 15° C. the fish tend to be unduly restless.

3. ALCOHOL, CHLOROFORM, FORMALIN

A number of experiments were run with alcohol, chloroform and formalin, substances that may be presumed to have an unfamiliar taste and smell to fish. In most of these five fish were placed in the apparatus and the experiment conducted as just described, but a series was also run in which single fish were used and all their movements recorded by the method of Shelford and Wells. Some typical results are given in Fig. 2, which also serves to illustrate the varied behaviour displayed by different fish with water running in at both ends; thus in the second experiment the fish remained motionless until the solution was admitted, whereas that in the fourth was exceptionally restless.

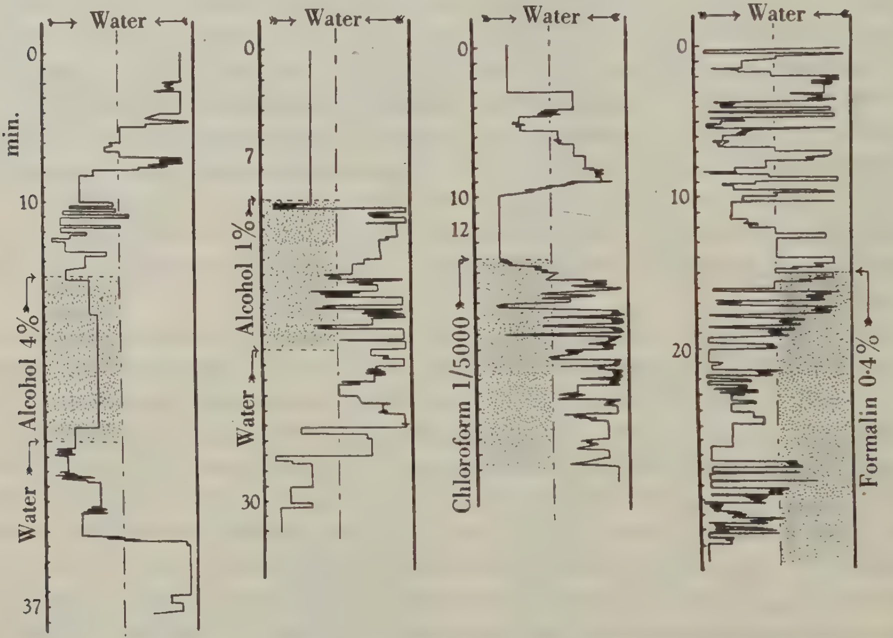

Fig. 2. The reactions of single fish to 4 and 1% ethyl alcohol, 1/5000 chloroform, and 0·4% formalin. Concentrations are v/v.

On running in 4% ethyl alcohol the fish is almost immediately intoxicated, its breathing becomes slow and irregular; later the fish falls over on its side and is incapable of movement so that a pseudo-positive reaction is obtained. On restoring the flow of water (26 min.) it rapidly recovers, at 35 min. is upright and breathing vigorously, in 38 min. is swimming almost normally. With 1% alcohol a typical negative reaction is shown; note the somewhat hesitant return to the left side of the apparatus on replacing the solution by water.

The results with chloroform resemble those obtained with alcohol. A typical negative reaction is displayed towards 1/5000 and 1/10,000 solutions, but high concentrations (1/2000) may anaesthetize or kill so rapidly that a 'positive' reaction appears. Formalin in concentrations of 0·1–0·4% (v/v dilution taking the concentrated solution as 40%) also gives a negative reaction with very obvious irritating effects.

4. MERCURIC CHLORIDE

A representative series of the results obtained with mercuric chloride is set out in Fig. 3. The reaction to a 0·003 *N* solution is violent; on running in the solution the fish make a frantic exodus to the water end, and thereafter, should a fish venture towards the solution, immediately it reaches the water-solution junction it moves jerkily with gobbling respiratory movements and retreats. An almost equally marked effect is produced by a 0·001 *N* solution, but at 0·0003 *N*, though the salt is highly toxic at this concentration (survival time 31 min.) the reaction is much slower, for it will be noted that 9–10 min. went by before all the fish departed, and the frantic swimming and gobbling respiration seen at the higher concentrations was no longer evident. A number of experiments were carried out at 0·0001 *N* with somewhat indefinite results. The fish would show a negative reaction if, under control conditions, they had shown no particular liking for either end of the apparatus, but when a preference for one end existed they would not leave the solution if run in on that side. One experiment was carried out at 0·00004 *N*, that shown in Fig. 3. For the first 30 min. no reaction developed, the fish kept swimming into the water but returned, swimming into the solution with no hesitation. Later a vague tendency to prefer the water zone developed, but by this time the fish were showing obvious signs of respiratory distress and the experiment was discontinued.

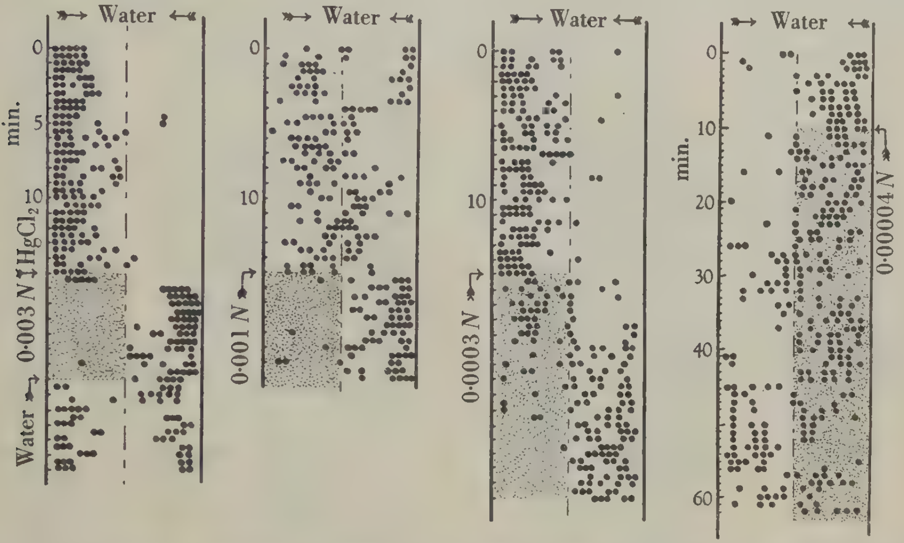

Fig. 3. The reactions of groups of five fish to 0·003, 0·001, 0·0003 and 0·00004 *N* mercuric chloride. *p*H of all solutions and tap water 6·6. Survival times at these concentrations (continuous immersion in the solution)—14, 22, 31, 100 min.

At the end of each experiment the fish were removed, washed in two changes of water and placed in an aquarium. A high proportion died within 24 hr., for mercuric chloride has such a rapid astringent or corrosive action upon the gill membranes that brief exposure to high concentrations may ultimately prove fatal.

5. ZINC SULPHATE

Eleven experiments were made with zinc sulphate solutions and four are recorded in Fig. 4. A prompt negative reaction is shown at 0·04 *N*, the fish almost immediately select the water zone and avoid the water-solution junction. Occasionally a fish enters the salt zone where it swims with great agitation until it reaches the water-solution junction; here it hesitates for a moment and then proceeds into the water.

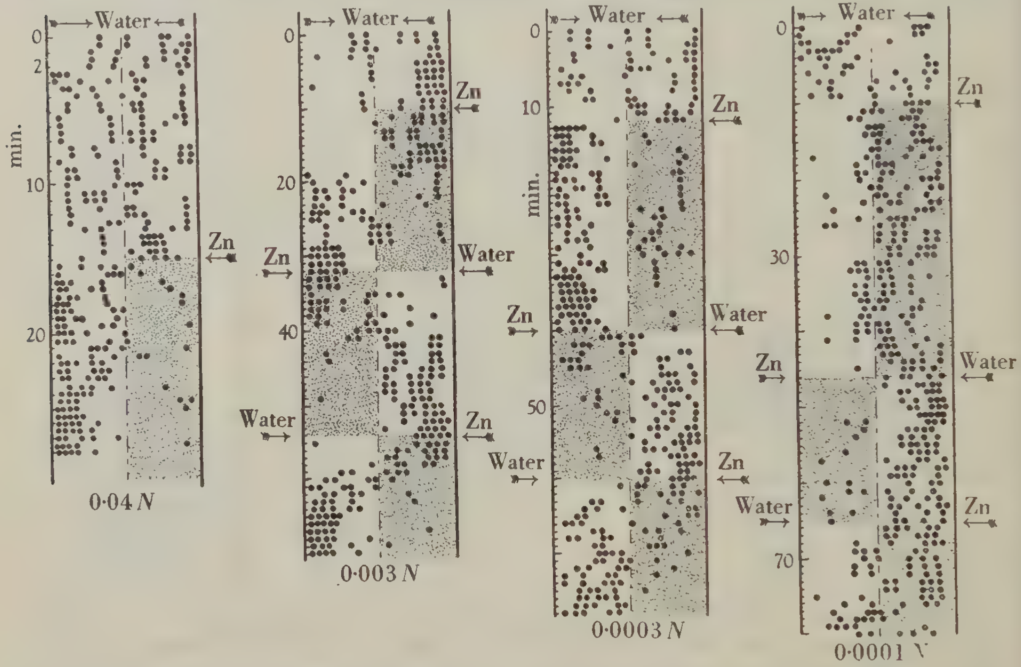

Fig. 4. The reactions of groups of five fish to 0·04, 0·003, 0·0003 and 0·0001 *N* zinc sulphate. *p*H of solutions 6·2, 6·4, 6·6, 6·6. *p*H of tap water 6·6. Survival times at these concentrations 85 min., 190 min., 7 hr., 15 hr.

At 0·003 *N* a negative reaction is evident but is much less prompt; the fish show little sign of discomfort and seem to discover the water end of the apparatus by trial and error. The result of reversing the direction of flow of water and solution are evident, and the improved response on the second reversal will be noted. Three experiments were run at 0·0006 *N*, and in every case a negative reaction was observed though it might take 18–20 min. to develop, and so experiments limited to 10 min. would indicate fish as positive to this concentration. At 0·0003 *N* (approximately 10 parts Zn/million) a negative reaction is still evident, though water-solution reversals are necessary to show it up clearly and the movements of the fish are very leisurely. A solution of this concentration has no taste, even when held in the mouth for some time, and cannot be distinguished from tap water. It is interesting to find that the fish can detect and avoid in a few minutes a solution in which they can survive for 7 hr. Bull (1937) has shown that teleosts can detect very small salinity differences; thus *Gasterosteus aculeatus* will develop a conditioned response to a salinity change of only 0·23 part/1000; but the same author (1930) records that when *Blennius pholis* is presented with an unfamiliar substance (trinitro-butyl-toluene) it fails to recognize it; at least over fifty trials failed to produce any conditioned response.

At 0·0001N (about 3 parts Zn/million) *Pygosteus* displays little reaction, but as Fig. 4 indicates, the fish do not become 'positive' to the solution and avoid the water, for the water-solution reversals at 46 and 65 min. produce no result. The survival time at this concentration is about 15 hr., and it is possible that the fish would shun this very low concentration if given sufficient time. At the end of each experiment the fish were transferred to aquaria and all survived.

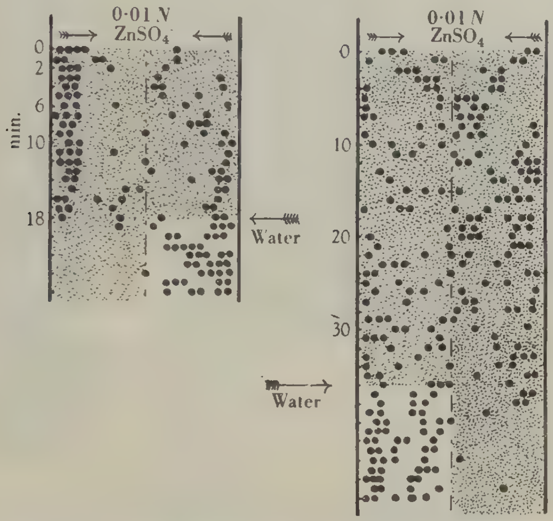

Fig. 5. The reactions of groups of five fish to water after 0·01N has been run in at both ends of the apparatus for 18 and 36 min. pH of solution 6·4. Survival time at this concentration about 100 min.

Experiments were also carried out to find whether *Pygosteus* can recognize and select water after a period of immersion in zinc sulphate solution. Two are recorded in Fig. 5. In the first 0·01 N $ZnSO_4$ was run in at both ends of the apparatus for 18 min., in the second for 36 min. In both cases the fish congregated in the water and avoided the solution almost immediately the change was made. In the second experiment the toxic process was well advanced, for marked respiratory distress had become evident.

No experiments have been carried out as yet to test whether repeated exposures to dilute concentrations of zinc sulphate will produce an improved negative response, but the writer hopes to pursue this line of investigation shortly. It may be noted that the concentrations to which *Pygosteus* will react when quite unfamiliar with metallic pollution may be exceeded under natural conditions, for the writer (1940*b*) has shown that in 1939 the Frongoch stream, a tributary of the river Ystwyth, contained 57 mg./l. of zinc in solution (about 0·0018 *N*). Trout occur in confluent tributaries, but whether they enter the polluted stream is not known.

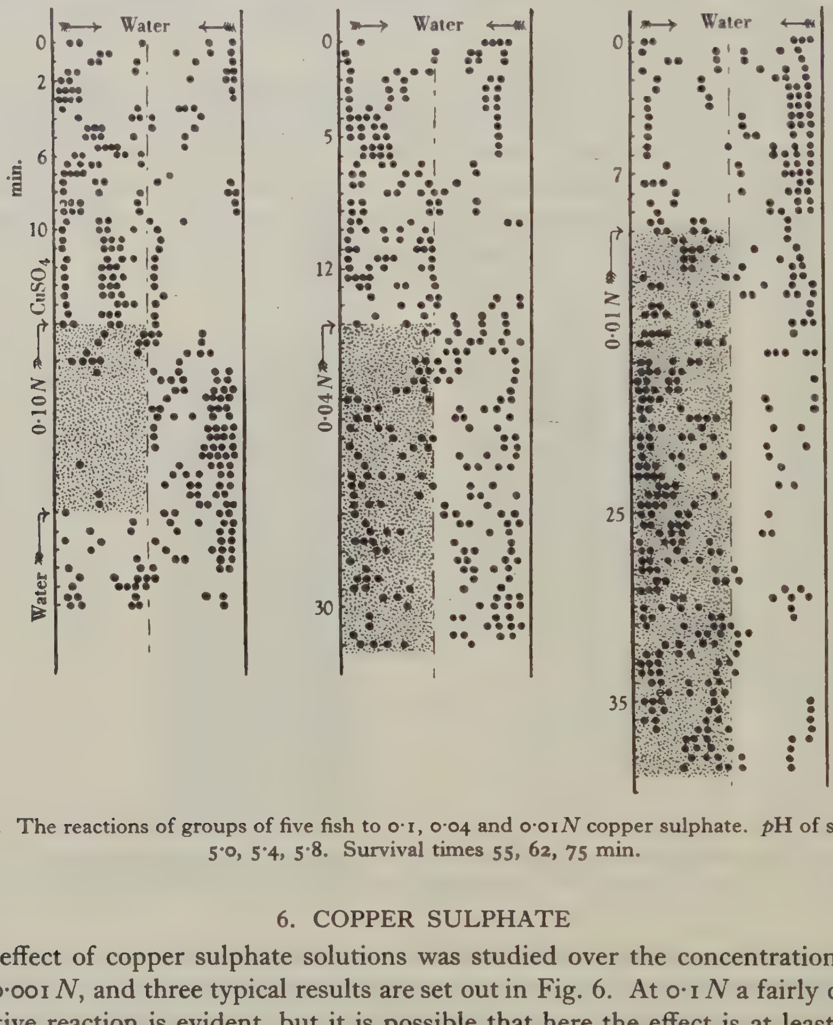

Fig. 6. The reactions of groups of five fish to 0·1, 0·04 and 0·01 *N* copper sulphate. *p*H of solutions 5·0, 5·4, 5·8. Survival times 55, 62, 75 min.

6. COPPER SULPHATE

The effect of copper sulphate solutions was studied over the concentration range 0·1–0·001 *N*, and three typical results are set out in Fig. 6. At 0·1 *N* a fairly definite negative reaction is evident, but it is possible that here the effect is at least partly due to the colour, acidity and high osmotic pressure of the solution. A 0·1 *N* solution cannot be brought to the *p*H of the tap water for copper hydroxide is precipitated on the addition of alkalis. At 0·04 *N* the reaction is vague; it will be seen that with water running in at both ends of the apparatus the fish demonstrate a preference for the left side; on running in the copper sulphate this preference is lost but the fish persist in swimming into the solution. At 0·01 *N* all sign of negative reaction has vanished; on the contrary, the fish congregate at the solution end of the apparatus, and a similar result is obtained at 0·001 *N*.

The explanation of this curious positive reaction was forthcoming when fourteen experiments had been run at 0·001–0·01*N* and the behaviour of the fish carefully watched. The fish are totally incapable of recognizing the solution and swim across the water-copper sulphate junction in either direction without the slightest hesitation. (See the recordings for single fish in Fig. 7.) In the solution they tend to become stupefied and rest motionless on the bottom; if they succeed in reviving a little and visit the other end of the apparatus the water seems to stimulate them, and they keep up erratic movements which eventually land them back in the solution where they spend another period of stupor. The charts in Fig. 7 show how the fish nearly always persist in swimming at the water end and remain still in the solution for periods of several minutes. In the five fish experiments at any particular time when the toxic process is well under way most of the fish are lying still in the solution while one or two are swimming in the water.

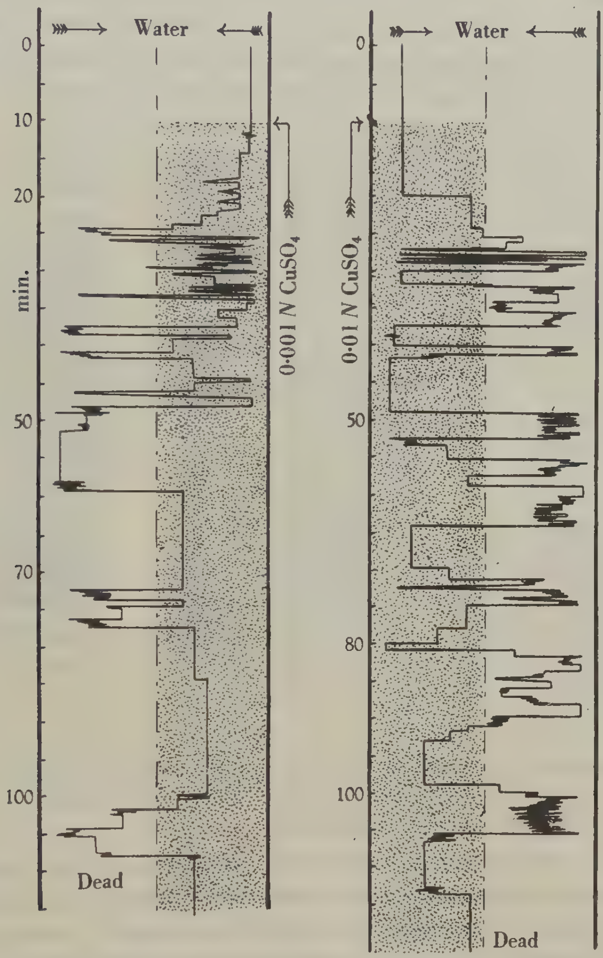

Fig. 7. The reactions of single fish to 0·01 and 0·001 *N* copper sulphate. *p*H of solutions 5·8, 6·6.

The 'positive' result therefore is not due to the fish deliberately selecting the solution, or becoming so intoxicated by it that they avoid the water. The whole explanation lies in the fact that they cannot perceive the toxic substance so that it acts as a trap. A parallel is to be found in the experiments of Jennings & Moore (1902) with *Paramecium* and *Chilomonas*; it was found that these organisms are apparently attracted to a drop of $M/180$ HCl, for they rapidly form a dense aggregation around it. Loeb (1918), reviewing their work, has shown that the explanation of this positive effect is that the drop of acid acts as a trap into which the organisms swim to be paralysed or killed, so that the density of the organisms around the drop increases until a dense ring of them is formed.

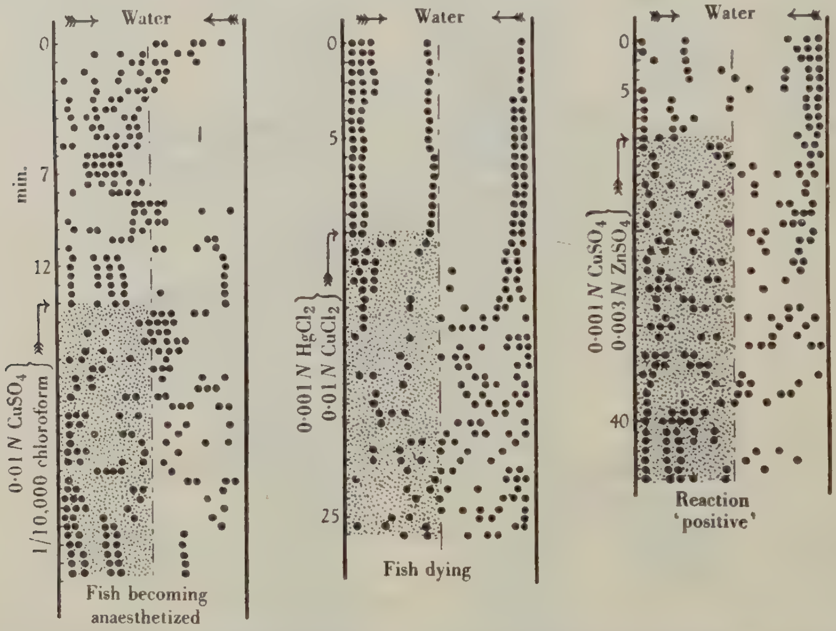

Fig. 8. The reactions of groups of five fish to 1/10,000 chloroform with copper sulphate added to a normality of 0·01; a $0{\cdot}01N$ $CuCl_2$ and $0{\cdot}001N$ $HgCl_2$ solution compounded in the same way; and a $0{\cdot}001N$ and $0{\cdot}003N$ $CuSO_4$ plus $ZnSO_4$ solution.

A $0{\cdot}01N$ solution of copper sulphate has a very distinct and highly unpleasant metallic taste, and it is interesting to find that fish cannot perceive it when they can detect such great dilutions of zinc sulphate. Furthermore, copper sulphate seems to destroy the stickleback's ability to distinguish other substances, for the usual negative reactions are not displayed to chloroform, mercuric chloride and zinc sulphate solutions to which copper salts are added. Thus Fig. 8 shows that a 1/10,000 chloroform solution containing copper sulphate produces no reaction and the fish become anaesthetized, and that copper chloride inhibits the reaction to mercuric chloride with the result that the fish are all dying after running the solution for 16 min. Copper chloride was used in this experiment as mercuric sulphate is an unstable salt. In the experiment with zinc sulphate and copper sulphate the reaction is 'positive'.

A number of experiments were also carried out in which the fish were immersed in $0{\cdot}01N$ copper sulphate for periods of 5–15 min., water run through the apparatus for 3 min., and then chloroform or formalin solution on one side. In every case either no negative reaction was evident, or it was much delayed.

7. GENERAL OBSERVATIONS

The present study does not aspire to be an exhaustive study of the reactions of fish to heavy metal salts, for it is obvious that many more remain to be tested, and there is no reason to suppose that other species of fish will react in exactly the same way, but certain conclusions can be drawn. It is obvious that with any particular substance the reaction of the fish depends on the concentration difference maintained at the water-solution junction. Secondly, it will be noted that the reaction displayed bears no relation to the general degree of toxicity of the salt, for zinc sulphate, a heavy metal salt of comparatively low toxicity, is detected at great dilution, while copper sulphate, one of the most toxic, is detected only at very high concentrations. Lastly, it appears that when we employ mixtures of substances the presence of one may impair or inhibit completely the reaction usually displayed towards the other when present alone, so that the study of the reactions of fish to effluents containing some variety of lethal substances becomes one of great complexity.

SUMMARY

An apparatus is described in which small fish (*Pygosteus pungitius* L.) are confined in a horizontal glass tube, half of which is filled with flowing tap water and half with flowing solution. Solution and water are very sharply differentiated, so that the concentration difference the fish encounter is known. The directions of flow can be rapidly reversed in order to check the result. The movements of the fish are recorded for 7–120 min., according to the degree of toxicity of the substance tested.

A negative reaction is shown towards 1% ethyl alcohol, 1/10,000–1/5000 chloroform and 0·1–0·4% formalin (% HCHO). High concentrations of these substances may stupefy the fish so rapidly that a pseudo-positive reaction appears.

A sharp negative reaction is displayed towards $0{\cdot}003$–$0{\cdot}001\,N$ mercuric chloride; at 0·0003 the reaction is delayed, at 0·0001 is indefinite, and at $0{\cdot}00004\,N$ solution, though of comparatively high toxicity, does not appear to be detected.

Zinc sulphate appears to be detected and avoided at concentrations at least as low as $0{\cdot}0003\,N$. This concentration is of comparatively low toxicity and may be exceeded in streams polluted by effluents from zinc mines.

Copper sulphate is only detected and avoided at extremely high concentrations ($0{\cdot}1N$). At $0{\cdot}04N$ the reaction is vague, and at $0{\cdot}01$–$0{\cdot}001N$ the fish swim into the solution where they become stupefied and lie motionless, so that the reaction appears positive.

Copper salts appear to impair or destroy the stickleback's ability to distinguish other toxic substances.

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DISTRIBUTION OF GLYCOGEN IN THE DEVELOPING SALMON (*SALMO SALAR* L.)

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(*Received* 10 *November* 1946)

(With Plates 2–4 and One Text-figure)

The presence of glycogen has been recorded in the eggs and larvae of teleosts as a result of histochemical methods, notably by Pasteels & Léonard (1935), who included the fertilized eggs of trout (*Salmo irideus*) in material used to test the efficacy of various fixatives. They reported glycogen in the blastodisc of the trout egg.

It has been pointed out by Needham (1931, **1**, 355) that results of histochemical observations upon glycogen in fish have not necessarily been confirmed by chemical estimations. Even in one of the rare cases quoted in which glycogen is supposed to have been estimated in fish eggs, it seems that a mistake has been made in translation. Fauré Fremiet & Garrault (1922) are reported, by Hayes & Hollett (1940), to have recorded 0·34% glycogen in the trout egg. What the former actually stated was 'Nous n'avons pas pu déceler la présence de glycogène dans l'œuf de Truite, mais nous avons trouvé une faible quantité (0·34 pour 100) de sucre réducteur que nous avons dosé en glucose'; which seems to mean that they found 0·34% glucose and no glycogen.

Information on the subject of glycogen content is less satisfactory for developing teleosts than it is for Amphibia. Attention has been concentrated upon development in the latter group through the study of Organization Centres. Methods for estimating small quantities of glycogen have been applied, and there is general agreement between results of histochemical and chemical investigations, e.g. Needham (1931, **2**, p. 1046), in spite of differences over the question of a glycogen decrease in the dorsal lip of the blastopore of Amphibia during invagination.

There is reason, therefore, for the application of recent improvements in histochemistry and quantitative estimations to the study of teleosts.

The developing eggs and larvae of the salmon (*S. salar*) have been chosen here for investigation by both methods. In 1941 sagittal sections were prepared from material in different stages of development and examined for glycogen. At this time attention was drawn to a paper by Hayes & Hollett (1940) which deals with the carbohydrate content of eggs and larvae of *S. salar* up to the end of the yolk-sac period. To obtain total carbohydrates, including glycogen, they used water extraction in a Soxhlet apparatus for 2–3 hr. Results obtained do not agree in certain particulars with the distribution of glycogen as seen in sections.

Their method is not well adapted for determining small quantities of glycogen. Carruthers & Ling (1934), for instance, do not regard water extraction of glycogen and total sugars as satisfactory for work on liver tissue with less than 4% glycogen.

Untoward circumstances prevented the carrying out of estimations by a modified Pflüger method until 1944–5, but these being completed, the results are included, for convenience, in the first part of the paper, although in point of time they were obtained three years later than the series of prepared sections. On the whole these results are in agreement with the distribution of glycogen as shown in the histological sections.

HATCHING AND REARING OF SALMON LARVAE

The larvae were kept in a hatching apparatus similar to that described by Moult (1939); main water, which is of good quality, was run through the trays.

Stages I–XII, for histological work, were obtained during the winter of 1941–2 from ova fertilized at the River Dee (Wales) Fish Hatchery on 21 November, and a second series, samples 1–12, for glycogen estimated in 1944–5, from ova fertilized there on 23 November 1944.

Varying conditions of water temperature during each season made it advisable to find a basis for comparing the rate of development in the two series.

A usual method adopted is the thermal unit, originally proposed by Wallich (1900), who states, however, that chief dependence should not be on the unit but on the appearance of the various stages.

In the present investigation the thermal unit was not found to be of any service. For instance, in 1941–2, the larvae hatched out at 615–632 thermal units, in 1944–5 at 897 thermal units.

Battle (1944) has noted similar discrepancies in the salmon. She states on p. 116 that 'the variation in the thermal units required to reach the same status in these (her own) two series, throws some doubt on their validity as criteria for determining comparable stages in embryos'.

Indeed, I have found the excellent drawings accompanying her paper much more useful in identifying various stages.

The advantages of drawings of easily identified stages has been recognized by Pelluet (1944), and a series he has produced for the salmon has been helpful.

In order to synchronize the two series dealt with in the present paper the following plan has been adopted:

Samples 1–12 (representing the estimations) have been plotted along the base line of Text-fig. 1 and spaced according to the number of days each sample was taken before or after hatching. Stages I–XII (representing the histological sections) have been put in the same figure, but the spacing of them is not related directly to the time scale. Each stage is placed in a position relative to the samples, which seems warranted by comparison of the development reached in the two series. To take the simplest example, stage VI followed immediately on the hatching out of the majority of the larvae in 1941–2. It corresponds, therefore, with sample 7, which was also taken immediately after the hatching in 1944–5.

For general comparisons the following data may be given:

Present series			Battle Figure (nearest)	Pelluet Stage (nearest)
Samples		Stages		
1	=	I	37	—
2–3	=	II –	38	—
3	=	III nearly	22–23	6
4	=	III – IV	24	8
5	=	IV +	25	—
6	=	V +	26–27	12
7	=	VI	34	—

Once the larvae are hatched the stages of development can easily be identified from Pl. 2, figs. 4–6 and Pl. 4, fig. 13.

CHEMICAL ESTIMATIONS

Preparation of Material

The amounts of glycogen in the embryos and in the yolk-sacs were estimated separately. The eggs or larvae were first weighed alive and then thrown into hot alcohol. This stopped enzyme action and also coagulated the yolk contents, so that the yolk-sac could be cut from the blastoderm or embryo without serious loss of material. In this separation a fair amount of yolk was included with the embryo, to make sure that no true blastoderm or embryo tissue was included under the heading 'yolk', particularly the liver which lies couched on the yolk.

The precaution actually caused a division into: (*a*) embryonic tissue plus a certain amount of yolk-sac, and (*b*) rest of the yolk-sac and yolk. A part of the protoplasmic vitelline membrane network was also included under yolk-sac and, after the anterior and lateral margins of the blastoderm had grown over the yolk, there would be included, also, ectoderm of the embryo and later mesoderm and endoderm. In fact the yolk in the salmon is finally enclosed by embryonic tissue which forms the sac.

Estimations

It is clear from the figures of Hayes & Hollett (1940) that the amounts of glycogen in salmon larvae are comparatively small, and it is necessary to adopt methods suitable to such conditions. About fifty larvae were taken for each of the earlier samples, but with an increase of glycogen later, numbers were correspondingly reduced or only part of the sample was used for final estimation.

Extraction and hydrolysis were carried out by the method of Good, Kramer & Somogyi (1933) with the following minor modifications:

(*a*) Two volumes of alcohol were added to the mother liquid of caustic potash.

(*b*) After preliminary dissolving in water and reprecipitation with alcohol, the material thus obtained was dissolved once more in water and neutralized to litmus with 1% sulphuric acid. Yolk precipitated by the neutralization was filtered with careful washings.

Determination of glucose, obtained by hydrolysis, was made by the copper-iodometric reagent of Somogyi (1937), devised for the estimation of small quantities.

The solution is practically reagent 50 of Shaffer & Somogyi with added sodium sulphate. The 200 g. of the latter per litre recommended by Somogyi seems to be excessive, since about 167 g. of material crystallized out from the solution under laboratory conditions of temperature. The decanted solution, when tested against known strengths of glucose, however, gave reduction equivalents very similar to those in the original paper. As an instance the reading for 0·5 mg. of glucose, after boiling for 20 min., was 4·68 c.c. of 0·005 *N* thiosulphate compared with 4·60 c.c. of 0·005 *N* thiosulphate.

In the samples the possibility of reducing substances other than glucose being present during boiling with the copper reagent cannot be dismissed. Young & Inman (1938) have reported small amounts of cystin and glucosamine in the dried egg capsule of the salmon. Although pyrocatechol, derived from glucosamine by boiling with caustic potash, reduces cupric copper, it is soluble in both water and alcohol and will therefore stay in solution and be discarded when the centrifuged tubes are decanted. Cystin may be less easily disposed of by the method of glycogen extraction adopted, but any effect it might have on the low alkalinity reagent of Somogyi would be at a minimum.

The specific action of amylase upon polysaccharides has therefore been used as an additional means of testing for glycogen in the tissues under examination. The sensitivity of enzyme action involves such factors as temperature and the purity of enzyme and substrate, together with incomplete hydrolysis (e.g. Sahyan & Alberg (1931) found that takadiastase at 38° C. for 4 hr., hydrolysed only 52 % of the original pure glycogen). Quantitative estimations, therefore, would need to rest upon a basis of further investigation.

Nevertheless, it is useful in the present work to demonstrate by the use of amylase, the presence or absence of glycogen in the various samples, as a further check upon the validity of the glycogen distribution as shown by estimations and in sections.

For this purpose, when the stage was reached in quantitative estimations for hydrolysis, the content of the centrifuge tube was first dissolved in 10·0 c.c. of distilled water; 5·0 c.c. of this solution was then pipetted into a second centrifuge tube, so that each tube contained one-half of the particular sample.

The original tube was dried off again and used for acid hydrolysis and subsequent glucose estimation. The second tube was also dried, and 5·0 c.c. of filtered saliva (1 : 10) + toluene was added before keeping in an oven for 24 hr. at 38° C. An advantage of using saliva is that whereas it hydrolyses glycogen, any galactogen which happens to be present will remain unattacked (May, 1931).

At the end of this time, 2 vol. of ethyl alcohol was added to the tube, so that any glycogen still intact, dextrins formed and any other material soluble in water and insoluble in 66 % alcohol, which had come through the various precipitations with 2 vol. of alcohol, following the original boiling with caustic potash, would be thrown out of solution again.

Any substance remaining in solution in the 66 % alcohol, therefore, would have been produced by the specific action of saliva, and it remained to identify the substance.

If a sample of pure glycogen is boiled in caustic potash and put through this process with saliva and subsequent addition of 2 vol. of alcohol, what is held in the final solution is not maltose, as might be expected, but glucose. This was established by the formation of typical glucosazone crystals when the solution was concentrated and boiled with phenylhydrazine hydrochloride and sodium acetate. Estimations of glucose showed the yield of glycogen to be low and variable, amounting from 10 to 33% of the original glycogen taken.

The glucosazone reaction was also obtained from salmon larvae by taking 150 larvae left over at the end of the experiment and submitting them to the same procedure. This was also true for a similar number of larvae that had been fixed in Bouin-Allen and preserved in 70% alcohol for a year. In view of the facts already given, the glucose could only have been obtained from glycogen originally in the tissues of the salmon larvae.

Table 1. *Glycogen (glucose × 0·927), mg. per larva*

	Days before or after hatching	Av. wet wt. per larva	Blastoderm or embryo			Yolk-sac and yolk			Total glycogen (mg. per larva)
			Av. wet wt.	Glycogen	Glycogen % wet wt.	Av. wet wt.	Glycogen	Glycogen % wet wt.	
Fertilization...	−49								
Sample 1	−44	151	21	0·029	0·14	130	0·027	0·21	0·056
2	−38	151	36	0·015	0·04	115	0·011	0·01	0·026
3	−30	152	42	0·014	0·03	110	0·038	0·03	0·052
4	−23	152	47	0·015	0·03	105	0·022	0·02	0·037
5	−15	152	33	0·010	0·03	119	0·024	0·02	0·034
6	− 9	155	34	0·023	0·07	121	0·026	0·02	0·049
7	+ 2	128	53	0·032	0·06	75	—	—	—
8	+11	128	45	0·044	0·09	83	0·012	0·01	0·056
9	+20	129	57	0·109	0·19	72	0·072	0·10	0·181
10	+25	135	83	0·141	0·17	52	0·087	0·16	0·228
11	+32	147	112	0·168	0·15	35	0·053	0·15	0·221
12	+39	177	—	—	—	—	—	—	0·319

The most satisfying procedure would have been to apply the glucosazone test to every sample of embryo and yolk-sac taken throughout the larval series. It was obvious, however, from the low returns given by the action of saliva upon pure glycogen, that in these samples small amounts of glucose would be involved, which would be insufficient to support the glucosazone reaction.

The Somogyi copper iodine reagent, therefore, was used instead as a qualitative test for the presence or absence of a reducing substance, after the use of saliva as outlined above.

Using filtered saliva as a blank, all results were positive, and from the evidence presented it may be inferred that the reducing substance was glucose. The lowest reading was the equivalent of 0·01 mg. glucose for half the amount of yolk-sac and yolk taken for sample 2.

The results of the glycogen estimations are given in Table 1. Although, as already explained, the division into blastoderm or embryo and yolk-sac is somewhat arbitrary, the average wet weight and glycogen percentages have been included in their appropriate columns.

The actual weight of glycogen is much the same in blastoderm and in yolk-sac for samples 1 and 2. It is greater in the yolk-sac in samples 3, 4, 5 and 6 and in the embryo in samples 8, 9, 10, 11 and 12, as might be expected, since the yolk is gradually disappearing and the embryo growing at its expense.

Nevertheless, there is an actual rise in the weight of yolk-sac glycogen in the later samples, which reflects a quickening in deposition.

The glycogen percentages on wet weight give a different picture. They are usually a little higher in the blastoderm or embryo than in the yolk-sac. Since the yolk itself adds much to the total weight of the yolk-sac and carries no glycogen in its main bulk, the percentage figures suggest that glycogen may, actually, be more concentrated in, or near, the embryonic tissues of the yolk-sac than in the embryo.

Results obtained by Hayes & Hollett (1940) for *S. salar* differ from the above in the following particulars:

They state that 'no glycogen was detected in the yolk-sac except on two occasions and then in such minute quantities—of the order of 0·01 mg.—that it might well be attributed to error'.

The first appearance of glycogen recorded by them was about 3 weeks after fertilization. Taking into account a slightly longer time between fertilization and hatching in the developing salmon of the winter 1944–5, this would mean an appearance about 5 days after sample 3 (1944) was taken, i.e. about the time of stage III.

From this it can be concluded that they found no glycogen in their samples which correspond to stages I and II of the present series, and their first record would be about stage III.

Further comparison between the two series can be made by examining the graphs of total glycogen per egg or larva included in Text-fig. 1. Hayes & Hollett based their results upon an egg of 130 mg. at fertilization, but this does not inconvenience the comparison.

The important difference is that glycogen was present in all samples from 44 days before hatching onwards, whereas the first record of Hayes & Hollett was 22 days before hatching.

While the present amounts are small and show little change in the embryo or the yolk-sac until 10 days after hatching, their series rises from zero, 28 days before hatching, through the time of hatching and onwards with two check periods, 6–10 days after hatching and about 32 days after hatching.

Both investigations agree in a rapid increase in amounts of glycogen between 20 and 39 days after hatching, the latter being the time of the last record in the present series, and this is the period of maximum fat absorption in developing salmon eggs (Hayes & Ross, 1936).

The finding of glycogen in the yolk-sac and of its early appearance in development agrees with the distribution of glycogen as seen in the histological sections to be described. It depended upon the use of methods which were devised to estimate small amounts of glycogen. It may be noted, however, that the steady increase recorded by Hayes & Hollett (1940) from 28 days before to 6 days after hatching, not

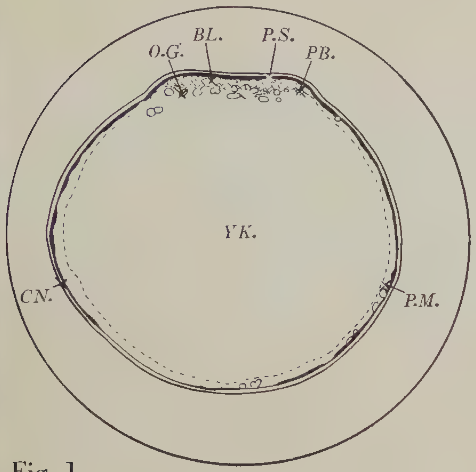


Fig. 1

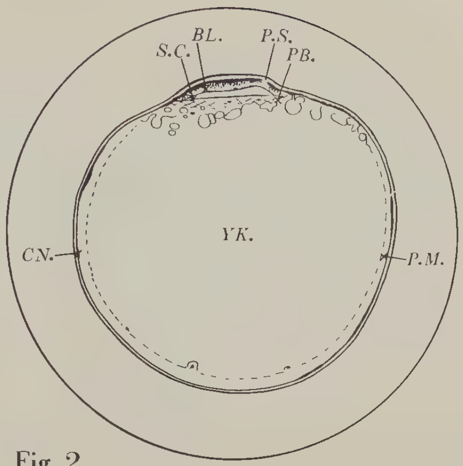


Fig. 2

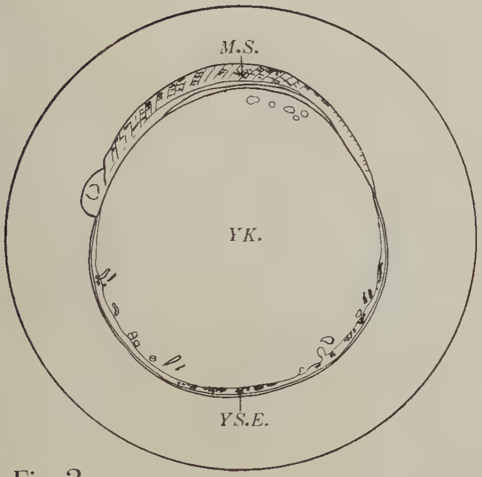


Fig. 3

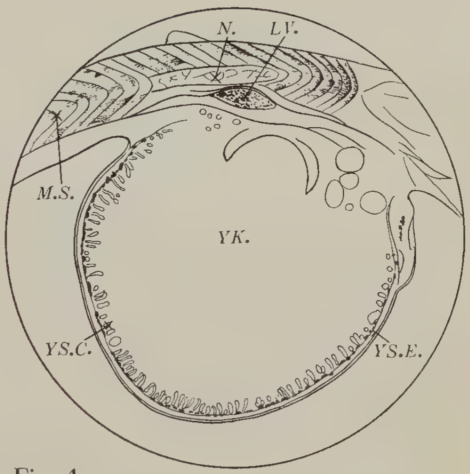


Fig. 4

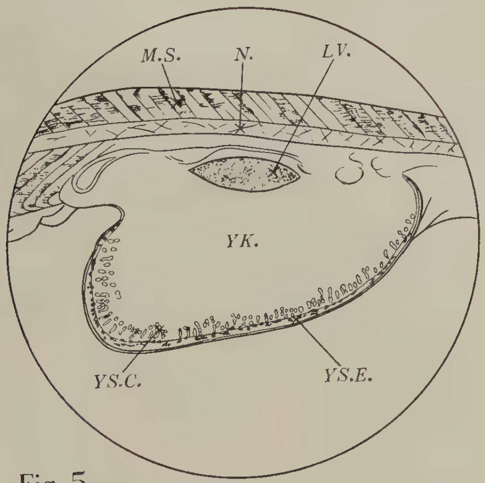


Fig. 5

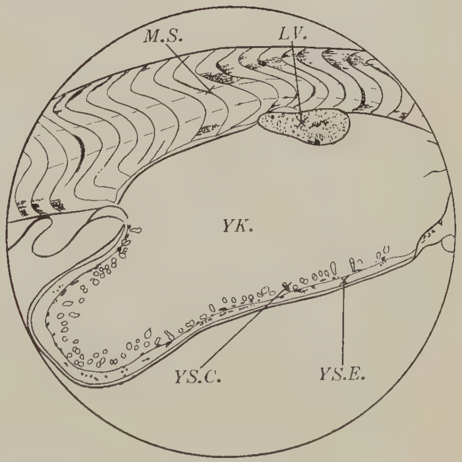


Fig. 6

DANIEL—DISTRIBUTION OF GLYCOGEN IN THE DEVELOPING SALMON

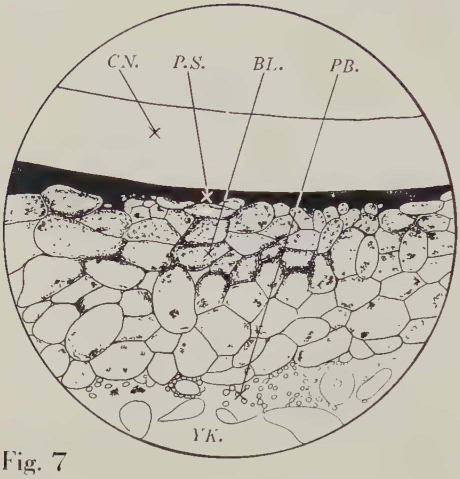


Fig. 7

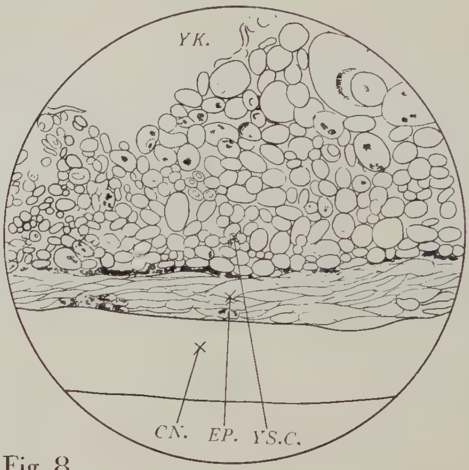


Fig. 8

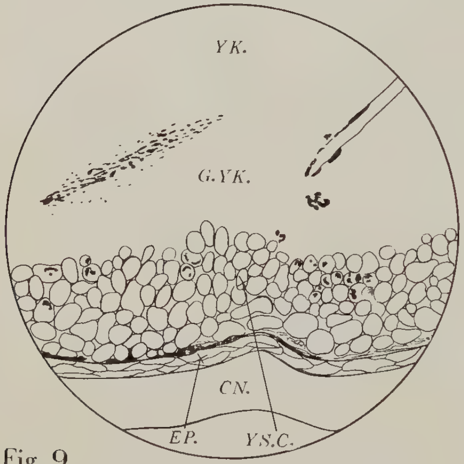


Fig. 9

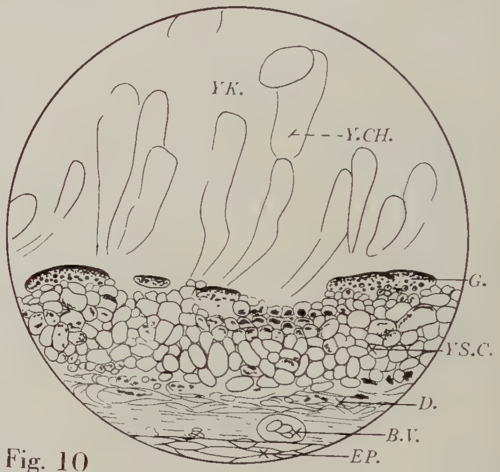


Fig. 10

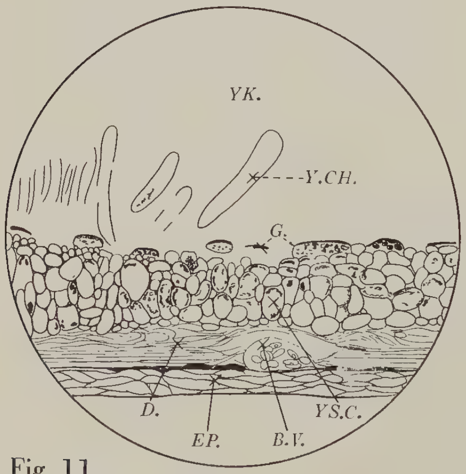


Fig. 11

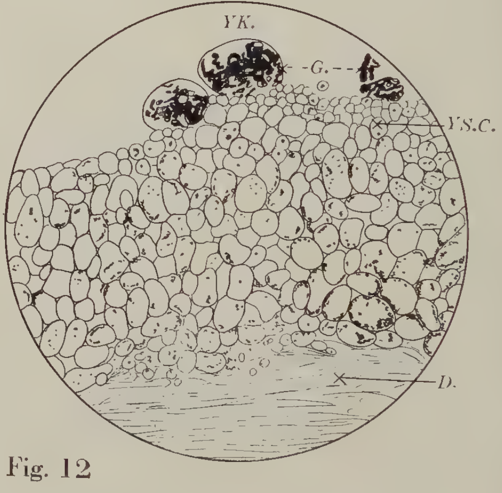


Fig. 12

DANIEL—DISTRIBUTION OF GLYCOGEN IN THE DEVELOPING SALMON

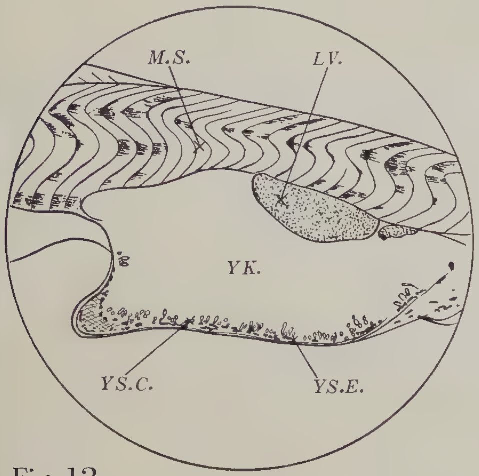


Fig. 13

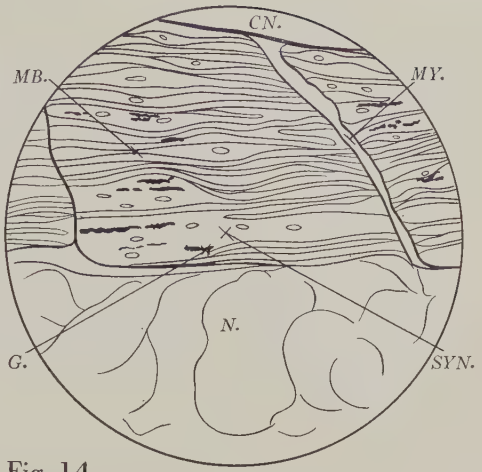


Fig. 14

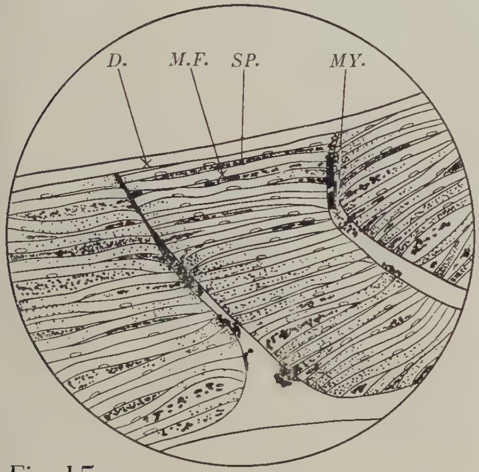


Fig. 15

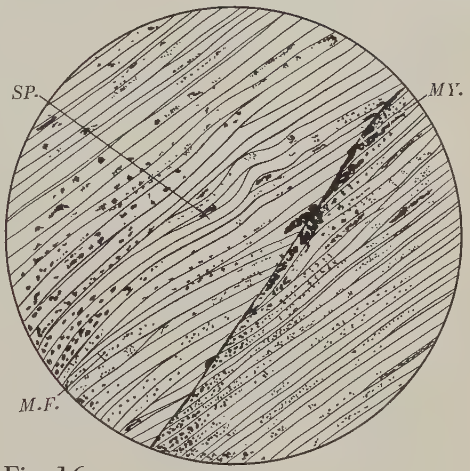


Fig. 16

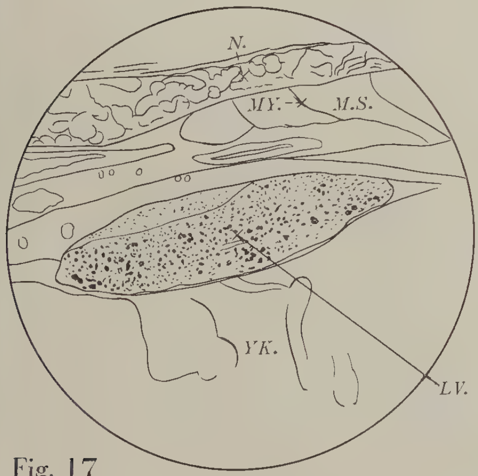


Fig. 17

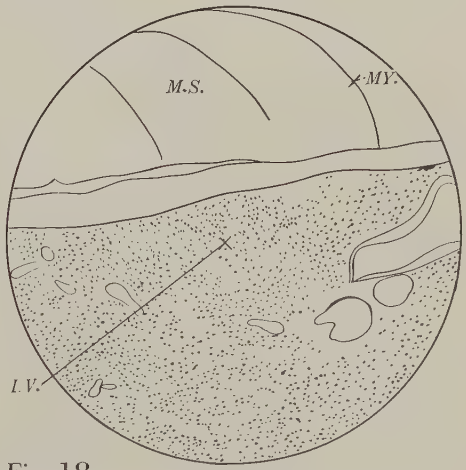
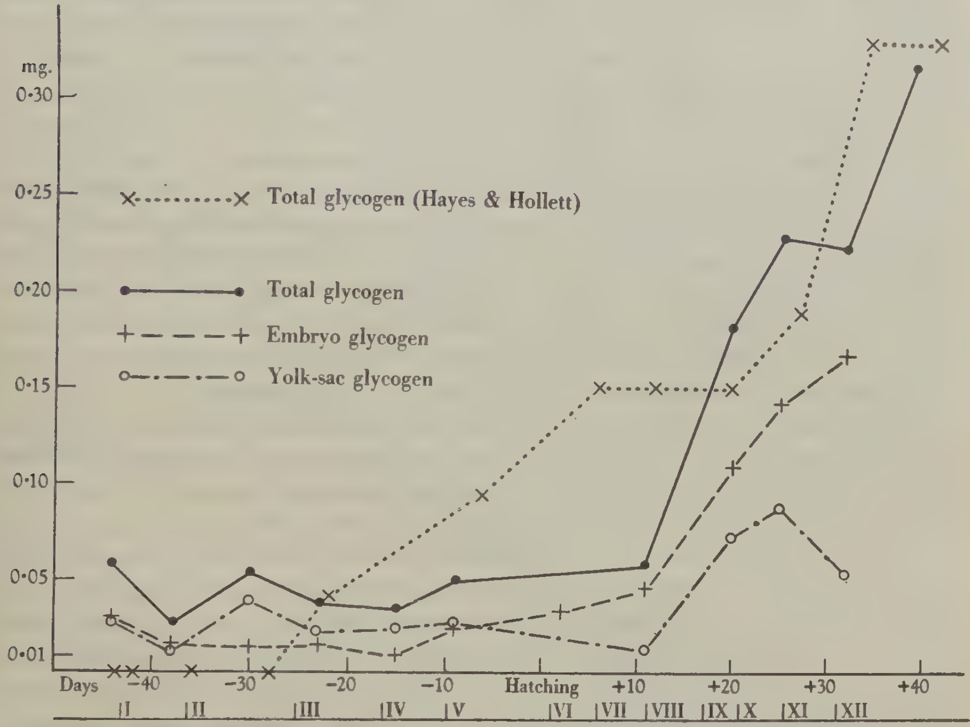


Fig. 18

DANIEL—DISTRIBUTION OF GLYCOGEN IN THE DEVELOPING SALMON

found in the 1944–5 series, covers the period from stage III to stage VI in the histological series, during which glycogen first appears in muscular tissue (stage III) and then in the liver (stage V).



Text-fig. 1. Samples 1–12 (representing the estimations) have been plotted along the base-line and spaced according to the number of days each sample was taken before or after hatching. Each of the stages I–XII (representing the histological series) is placed in a position relative to the samples which is warranted by comparisons of the development reached in the two series.

DISTRIBUTION OF GLYCOGEN IN TISSUE

Histological Methods

Sagittal sections were obtained which included the so-called animal and vegetable poles of the fertilized egg, and in later stages they cut through the longitudinal axis of the developing larva. Sections taken in the median vertical plane were always used for obtaining the figures, although lateral ones were also used for examination.

Material was fixed in Bouin-Allen or picro-dioxan solutions, upgraded through *N* butyl alcohol in both cases, and imbedded in paraffin wax m.p. 58° C., to which 1% of ceresin had been added. Both fixatives, used in this way, gave similar pictures of the distribution of glycogen in stained sections.

Each wax block was shaved down until a thin slice had been removed from the imbedded tissue and then left overnight in a mixture of 70% alcohol and glycerine (9 : 1), or in Mollifex (B.D.H.) for yolk softening. After this treatment it was possible to cut a number of serial sections including the yolk, before the splintering of the latter gave warning that further immersion was necessary. The sections were cut comparatively thick (about 10μ).

With the above methods the chief technical difficulty was not concerned with the cutting of complete sections, but with sticking them to the glass slides before staining and mounting in Canada balsam. Although several methods were tried besides the usual routine of using egg albumen, the only successful one was to immerse the slides and sections in $\frac{3}{4}\%$ celloidin. If required this was finally removed by acetone before mounting.

The staining methods used for demonstrating glycogen were: (*a*) Lugol's iodine-potassium iodide solution (strong formula), the sections being counterstained with alcoholic light green. (*b*) Best's carmine. (*c*) Bauer's reaction, using chromic acid followed by Schiff's reagent. This is a particularly useful method because it provides its own counterstain, general tissue being blue in contrast with the red-violet colouring of glycogen. The deep brown stain of the iodine, the brilliant red colour of Best's carmine and the characteristic colour reaction of Bauer act as a check upon each other when the distribution of glycogen is being considered.

No less important is the use of control sections, prepared by immersion in warm water and afterwards leaving for at least half an hour in saliva at 40° C. A clear solution of salivary amylase may be obtained by rinsing out the mouth with about 10·0 c.c. water at a time, and filtering the product before use.

The check sections should be prepared and examined with great care after they have been stained and mounted. The almost complete absence of deep-staining reactions following this procedure suggests a thorough removal of glycogen from sections. It is known that hydrolysis of glycogen to maltose (or later glucose) by salivary amylase is not complete. It can only be assumed that glycogen, and dextrins formed, are freed in some way from the action of the fixative so that they are no longer insoluble.

It is only possible to make general statements about the distribution of glycogen in sections. Magnification under the microscope tends to give a disproportionate idea of the actual quantity that is being examined. Further, the stained material may not be pure glycogen. The latter may be adsorbed on the surface of other material, or even form a loose complex with it. Examination of check sections, in which there is no distinctive staining reaction, is useful in revealing such conditions. A good example is found in larvae at stage V. Here there are inlets in yolk lying close to the yolk-sac cells. Discrete material in these inlets is heavily stained after iodine, Best's carmine and Bauer. In hydrolysed sections there is some unstained substance still present in the position marked in other sections by staining reactions.

Such conditions are found in subsequent stages and are very marked in stage IX, where, in addition, granular material is left behind in those places on the edge of the yolk cells where, apparently, there had been solid clots of glycogen.

Distribution of Glycogen

The three main centres of glycogen concentration as seen in stained sections are the liver, the muscular system and the cells lining the yolk-sac.

Glycogen has a wider distribution in the tissues; it appears, for instance, in the branchiae, but observation has been concentrated upon the three centres named, and these are examined in turn through the various stages of development, except stages I and II, which will be dealt with in their entirety since, in them, the tissues are not yet differentiated.

Stage I (Pl. 2, fig. 1; Pl. 3, fig. 7). At this stage the fertilized egg is enclosed by a tough outer membrane or chorion. Inside this, and spreading over the yolk, is a protoplasmic vitelline membrane. Between the two membranes is a perivitelline space, which also overlies a multicellular blastoderm resting on periblast at the animal pole. The chorion hardens when in contact with water and, according to Bogucki (1930), it is then impermeable, or pratically so, to colloidal solutions. He further states that the yolk releases colloidal substance into the perivitelline space. The fluid there is slightly opalescent and is said to contain protein. Certainly it has a strong reaction to stains used for glycogen, so that in section the perivitelline space shows up as a deep brown or red continuous band, according to the staining method used. A similar deposition of glycogen is apparent in the fertilized frog's egg; Needham (1931, **2**, 1044) states that it is partly eliminated into the perivitelline space and almost wholly used up by cells of the embryo before gastrulation. As for the origin of this glycogen in stage I, it may come from the yolk, as suggested above, but some glycogen is already at the periphery of the yolk in unfertilized salmon ova.

Glycogen is also concentrated in the tightly packed blastoderm cells. It is granular in appearance and concentrated towards the cell walls, so that in certain areas the staining reaction marks out the pattern of the cells.

Cells nearest the chorion contain most glycogen, so that, on the whole, there is a gradation in amount from the cells adjacent to the periblast to the heavily stained contents of the perivitelline space. There are also small granules of glycogen in the protoplasm of the underlying periblast.

Stage II (Pl. 2, fig. 2). There is the beginning of blastoderm differentiation in the position where the dorsal lip and the primitive endoderm will appear, but the blastoderm is still a number of cells deep in sagittal section, and the deposition of the glycogen is much the same as in stage I except that the perivitelline space is less deeply stained. Conditions round the yolk have not changed except that stained granules appear in certain spaces that seem to be formed by local shrinkage of yolk from the vitelline membrane.

Deposition of Glycogen in the Muscular System

It is to be considered that the distribution hereunder described in the later stages is that in larvae which have taken violent action before being caught for fixation.

Stage III (Pl. 4, fig. 14). There has been rapid development between stages II and III. The embryonic mesoderm is already separated into segmentally arranged compartments or myotomes, and certain of the derived myoblasts have started to divide and form elongated cells which span the length of the myotome. The myoblasts assume a syncytial character, and the small amount of glycogen that is present appears as isolated granules or streaks across a syncytium. These streaks do not span the myotomes in many cases, but they are elongated and therefore give the impression that they are connected with the formation of muscle cells, if not actually contained in them.

Stage IV. Although sections prepared from tissue fixed in silver nitrate at this stage show striped muscle fibres in certain restricted areas, the site of the muscular system is occupied mainly by elongated cells containing large nuclei and stretching from one myoseptum to the next.

In glycogen-prepared sections there are stained streaks, made up of closely packed granules, which seem to be in elongated muscle cells. Where these have formed such granules also appear in a more broken up state in the intervening sarcoplasm, where this is showing, but not in the myoblasts.

The embryo can now move with some vigour. It would appear from stages III and IV that glycogen is present in muscle tissue before the striped muscle fibres have been laid down.

Stage V (Pl. 4, fig. 15). Now the muscular system is complete in the portion of the mesoderm dorsal to the notochord. There are also striped muscle fibres ventral to the chord and lying close to it; more ventral still there remain elongated muscle cells mingled with striped fibre. The embryos are capable of strong movements within the egg capsule at this stage.

In section, glycogen is seen to be packed in the connective tissue of the myosepta (which is also a storehouse for fat) and in the myotomes, particularly near these septa. It is concentrated in the sarcoplasm between the muscle fibres, but also present as finer granules, in the fibres, particularly at the posterior end of the myotomes.

Stage VI (*just hatched*). The system of striped muscle fibres is finally established. Under the highest powers glycogen is found as very fine grains inside the fibres, although it also lies between them. It is concentrated towards the parts lying nearest to the myosepta, as in stage V. The septa, themselves, are also packed, so that the divisions between the myotomes are shown up clearly as a deeply stained pattern under the low power of the microscope.

Stages VII and VIII. There is glycogen in the septa between the myotomes, particularly near the dorsal surface of the embryo. It is also present in the sarcoplasm between muscle fibres, but seems to be less densely packed than in stage V, although it is concentrated in and between the anterior and posterior ends of the muscle fibres near the myosepta. The granules inside the fibres are always smaller than those outside.

Stages IX–XII (Pl. 4, fig. 16) are little changed, except that glycogen is more generally distributed in the muscle fibres, which have grown so that there is little intervening sarcoplasm. Where this occurs, however, there are still deposits of glycogen.

To summarize, glycogen is early present in embryonic tissue, when fibres are being laid down in the myoblasts, and it is present in the muscular system throughout the series. In earlier stages glycogen is more concentrated in the sarcoplasm than in the muscle fibres but this condition is reversed later, except in the areas which lie near the ends of the fibres and therefore adjacent to the myosepta. It is here and in the connective tissue septa that glycogen is most heavily concentrated, and this power of retention by the septa is shared, to some extent, by the subcutis which is continuous with them. The deposition may be so heavy that it is possibly not confined to the connective tissue cells.

Glycogen in the Liver

According to Battle (1942) the liver anlage arises early in the development of *S. salar*, when the embryo is about 4·0 mm. long. The blastoderm at this stage has overgrown slightly more than three-quarters of the yolk sphere.

In the series under discussion the anlage was first found in a larva 6·0 mm. in length (stage III) and near it, in one or two sections there were small granular bodies which stained deep brown with iodine.

The liver is certainly present in stage IV, but this was revealed in material that had not been fixed to retain glycogen.

Stage V. It was not until stage V, therefore, that the staining reactions for glycogen showed this substance in quantity in the liver cells, although it may have been there earlier, and it was found in all the subsequent stages examined.

Stage VI (Pl. 4, fig. 17). The liver is now conspicuous and lies deeply couched on the underlying yolk. The central portions of many of the liver cells are practically filled with glycogen, which is probably at its highest concentration at this stage.

Stage VII. The distribution of the glycogen is much the same as in stage VI except that it is somewhat less concentrated in the cells. It is not possible to say whether glycogen is in the cytoplasm only or in cell nuclei as well.

Stages VIII–XII (Pl. 4, fig. 18). There is little change in these later stages except that there is not the same amount of packing in any particular cell, so that glycogen appears to have a more even distribution throughout the organ.

Differences in the general appearance of the liver glycogen in stages VI and XII may be studied in Pl. 4, figs. 17 and 18. During the period covered by stages V–XII an increase in glycogen, as recorded by Hayes & Hollett (1940), would not be due to additional amounts in individual cells, but it would be consistent with the steady increase in the size of the liver itself, as outlined by Battle (1942).

Observations do not agree with Hayes & Hollett (1940) about the time of appearance of glycogen in the liver. In their Table I is given a first estimation of 0·01 mg. liver glycogen 6 days after hatching, but staining reactions are strong in stage V of the present series and very similar in appearance to those in later stages, where it is agreed by them that glycogen is present.

Now stage V was reached at least 8 days before hatching, and since the times between fertilization and hatching are about equal for the two investigations, the position of stage V in developmental time would be much the same in both cases and 14 days before the date given by Hayes & Hollett (1940).

The source from which the liver glycogen has its origin may be considered. The possibility that it came from ingested food may be ruled out because, according to O'Brien & Moult, the oral plate does not break through until the embryos are

9·0 mm. long and the oesophagus is not capable of passing food until they are 25 mm. long. In short, there could be no feeding, in the ordinary sense, until the last stage examined in this series.

Hayes & Hollett (1940) have demonstrated that glucose is present from the time of fertilization until the yolk-sac has disappeared and that it is certainly present in both yolk-sac and embryo from 12 days before hatching until 76 days after hatching. Although there is synthesis of glucose from fertilization until the disappearance of the yolk-sac, this is accompanied by a falling away in the total quantity, particularly in the yolk. They suggest that as the small embryo uses up glucose, it is replaced by simple diffusion from the yolk.

It may be that glucose is able to diffuse in this way through the capsule of splanchnic mesoderm which separates the liver from the adjacent yolk, and that from this source glycogen is formed in the liver cells, the energy for such transformation being derived from the glucose. The present investigation can throw no light upon the presence of such a mechanism.

A more obvious connexion between liver and yolk has been recorded in the sea bass (*Serranus atrarius*) by Wilson (1889), in the perch (*Perca fluviatilis*) by Chevey (1924) and in Salmonidae by Portmann & Metzer (1929).

As larval development proceeds, the outline of the liver next to the yolk becomes blurred, the dividing layer breaks down and liver cells in this region begin a direct absorption of yolk material.

In Salmonidae Portmann & Metzner found that in water at 7–10° C. there was no direct connexion in early stages, but that 30 days after hatching complete contact had been established between yolk and liver. In Fig. 4 of their paper liver cells are shown ingesting yolk material.

Examination of sections of *Salmo salar* shows that there is no suggestion of direct contact between liver cells and yolk, except in the last stage examined. Even here, 43 days after hatching, with temperatures 7–12° C., there is strong encapsulation of the liver, except in one localized area.

Here the splanchnopleura is very much attenuated and the adjoining yolk more vacuolated, but there is no suggestion of yolk absorption by liver cells as depicted by Portmann & Metzner (1929).

During the greater part of the development under review, therefore, there is no histological evidence that liver cells receive material, such as glycogen, by direct absorption from the yolk.

In teleost larvae the yolk, in fact, is isolated from the digestive tube, except under circumstances as mentioned above, a condition which is unlike that of vertebrates in general. Another characteristic of teleostean development is the rich supply of blood vessels which anastomose and form a complete network about the yolk-sac. It has generally been assumed, therefore, that food material is conveyed from the yolk to the embryo by means of these blood vessels. Statements to this effect have been made, for instance, by Jenkinson (1909) and by Ryder (1882). The latter claims to have observed yolk being absorbed into the circulatory system, but the evidence does not seem to be conclusive.

These blood vessels begin to develop early, before the head fold of the embryo is formed. Movement of the heart is indicated in the salmon before stage III is reached. By the time the liver diverticula arise, the blood system of the organ is connected directly with the yolk circulation which is venous throughout embryonic life, even though in later stages, before complete absorption of the sac, a direct portal vein is formed between liver and heart.

The system may well be a means of absorbing food material from the yolk and distributing it to the embryonic tissue, particularly the liver. In transverse section, however, the veins are seen to be superficial in position. Between them and the yolk, endoderm, as well as mesoderm, has passed out and enveloped the yolk. It is only through these layers of cells that any yolk absorption by the sac circulation can take place.

Glycogen in Yolk and Yolk-sac Tissue

In early stages after fertilization the yolk is homogeneous, but when stage IV is reached, there are indications that the more superficial part of the yolk is undergoing changes, which are reflected by a difference in appearance after fixation.

Notably in stage V this 'rind' has assumed an open, almost granular texture (Pl. 3, fig. 9). Thus modified the yolk reacts to staining in a different manner. It stains much more deeply with eosin and very intensely with aniline blue. It is equally well marked off from the rest of the yolk by a lighter blue belt in the general tissue stain that accompanies Bauer's reaction.

Another indication that the globulin in the outer portion of the yolk is undergoing change is the formation of inlets or rifts, so described because that is how they appear in sections and as such are figured in the plates. They probably represent a system of tunnels formed in the yolk periphery and may be numerous, but rarely occur in yolk adjacent to the embryo.

Yolk inlets are present after fixation of larvae by Bouin-Allen or picro-dioxan. They are present, also, in larvae fixed in formalin and subsequently sectioned by freezing microtome, but are less conspicuous. These latter sections also show rounded fat globules enveloped in the yolk.

In larvae of stages I–IV fixed for glycogen, fat globules have disappeared, but rounded vacuoles mark their position and show an early distribution over the periphery of the yolk, with a subsequent massing immediately below the periblast underlying the developing embryo.

In later stages inlets and fat vacuoles may be present in close proximity, and they look quite distinctive when seen in sections. Moreover, the fat vacuoles begin to concentrate under the embryo, and few are left round the yolk-sac when the inlets are increasing rapidly to a maximum in stage VIII.

The presence of these inlets near the yolk-sac cells in stages IV–XII may be taken as an indication that absorption by these cells is taking place as the yolk diminishes in size. This absorption is accompanied by the deposition of glycogen.

The Yolk-sac Envelope

From stage III onwards there are three distinct cellular layers which make up the yolk-sac envelope. First an outer epidermis of typical stratified squamous cells from which, after hatching, gland cells extrude.

Then there is an intermediate layer which takes the form of a dermis. It consists of an inner stratum of fibro-elastic tissue, and an outer one with interlacing bundles, mainly parallel to the outer surface of the envelope. It is this layer, immediately below the epidermis, which contains the blood vessels of the yolk-sac venous system. In parts of the sac, the dermis may almost disappear, as represented in stages III and V (Pl. 3, figs. 8, 9).

The inmost tissue consists of comparatively large and sometimes quite irregularly shaped cells which may form compact layers. The width of this cellular band varies a good deal and may be reduced to one or two cell layers by the intrusion of fat vacuoles.

It is not known if they are entirely endodermal in origin, and so they will be referred to simply as yolk-sac cells.

Stage III (Pl. 3, fig. 8). In stage II the anterior and lateral margins of the blastoderm have grown over the yolk and practically covered it. The germ layers in the embryo have already differentiated and the ectoderm is continuous with the yolk-sac envelope as layers of elongated cells with the walls hardly delimited, except at the outer edge lying near the chorion.

The rest of the sac lining in fig. 8 consists of loosely knit cells, some of them large, which present a foam-like structure in which it is not possible to distinguish mesoderm from endoderm cells. This tissue forms a thin layer for the most part, but in places it widens and projects into the yolk, when it has the deposition represented in the figure.

There is evidence of glycogen. It is deposited between the larger inner cells, and the epidermis and is present also in certain epidermal cells. The inner mass of cells has small amounts of glycogen which apparently lie both inside and between the cells.

Stage IV (Pl. 2, fig. 3). Cells on the edge of the yolk are now more uniform in appearance, and certain of them contain stained granules of glycogen which are concentrated towards the centre of the cell. There are also small deposits of glycogen in yolk near to these yolk-sac cells.

Stage V (Pl. 3, fig. 9). There is glycogen in certain of the yolk-sac cells. A good deal of cell proliferation is taking place next to the yolk, and there are heavily stained granular streaks of glycogen which have the appearance, in section, of being imbedded in the yolk or, more usually, lying where there are inlets or rifts in the yolk. Reference has already been made to the disturbed superficial area of the yolk at this stage.

In addition there may be deposits of glycogen between the yolk-sac cells and the epidermis, in regions where the dermis may have almost disappeared (as in fig. 9).

Stage VI (Pl. 2, fig. 4). In this stage, taken shortly after hatching, the inlets stretching from the peripheral cells are more pronounced and glycogen may occur near the cellular regions at their bases. There are many such concentrations, however, that do not correspond to an inlet. The concentrations all lie near to yolk-sac cells, and although they may be separated from them by yolk, they are usually found in restricted areas which are bounded on one side by yolk-sac cells.

There are also small amounts of glycogen in the dermal layer.

Stage VII (Pl. 3, fig. 10). In sections the specific stains for glycogen appear as bands of colour in the yolk-sac cells. The colour is concentrated towards the centre of the cells in such areas, but there is also a general diffuse staining which almost obscures the cell outlines.

In addition there may be intensive staining between cells and yolk; such deposits do not usually lie at the bases of the inlets and may be adjacent to clusters of small yolk-sac cells. In the latter case the cells are separated from the main glycogen deposit by an area of attenuated yolk, which contains open-spaced granules and, nearer to the glycogen, they are closer spaced. All the granules stain deeply for glycogen but are not entirely composed of it, since they are still present as unstained bodies after the glycogen has been removed.

In other cases the yolk-sac cells which border such an area are of normal size, or the area, with its granules, may be pinched off into the yolk. (These gradations are shown in fig. 10.)

Stage VIII (Pl. 2, fig. 5). The inlets near the edge of the yolk are now so numerous that they may join up and run into each other. Some of them show a staining reaction for glycogen at the inner (i.e. deeper) end.

There are also oval or pear-shaped areas in the yolk, which may be easily distinguished in section. They lie on the edge of the yolk-sac cells or may actually intrude into them. These areas are similar to those mentioned in stage VII; they may be partly empty but always contain glycogen either diffused or as granules. Some of the areas are practically imbedded in the sac cells. In such cases they may not be much bigger than the cells themselves and are completely filled with stained material after iodine or Best's carmine.

There is also a considerable amount of glycogen in the yolk-sac cells. The staining is very dense in the cells, or there may be stained granules scattered just inside the membrane.

Stage IX (Pl. 3, fig. 11). Although in stage IX the inlets are very much decreased in numbers, the general disposition of glycogen is similar to that of stage VIII. The boundaries of glycogen containing areas on the edge of the yolk-sac cells are partly formed by the cells themselves. These, in most cases, show no diminution in size, but in cases where they are smaller, it is possible for yolk to intrude between the cells and the area.

Further, any line of demarcation may disappear so that apparently nothing is left in the yolk but a streak of glycogen.

There are, also, large amounts of glycogen in the yolk-sac cells, and in the dermis along the inner epidermal cells.

Stage X (Pl. 2, fig. 6). Deposits of glycogen are less evenly arranged. They are wider and deeper than in the above stage, but there are fewer of them. In addition, there is a more scattered distribution along the border of cells and yolk as well as in the dermis.

Stage XI (Pl. 3, fig. 12). Granules which show a glycogen-staining reaction in areas adjacent to yolk-sac cells are comparatively large in size. They are not large enough, however, to be confused with certain small yolk-sac cells that occur near these granular deposits. Deposits of glycogen are found in the yolk-sac cells, as depicted in fig. 12, and there is also a general distribution of glycogen in the dermis round the blood vessels, when these are present.

Stage XII (Pl. 4, fig. 13). The distributing of glycogen is much the same as in stage XI.

In brief, the presence of glycogen in the yolk or on the edge of the yolk-sac cells can only be reviewed here as spatial relationships revealed by the histological preparations.

In stages IV and V (Pl. 2, fig. 3 and Pl. 3, fig. 9) such glycogen appears in the open texture of the more superficial part of the yolk, but removed somewhat in space from the yolk-sac cells, so that it may have arisen independently of the activities of these cells.

There is known to be a supply of glucose in the yolk (Hayes & Hollett (1940)), and it is possible that the necessary mechanism for transformation may also be there.

In stage VI and onwards, however, deposits are always near the boundary between yolk and yolk-sac cells, and therefore more obviously bound up with the latter. In these later stages there are areas of the yolk lying in contact with the cells, which may appear, in extreme cases, as spaces containing only glycogen, but which more usually contain granular bodies of different sizes.

These areas are large compared with the cells and have no direct connexion with the yolk channels or rifts, already mentioned, which occupy a more interior position in the yolk; nor are they to be confused with fat vacuoles that occur in the same vicinity.

The outline of the area which borders the cells is made up of the walls of these cells and is, therefore, somewhat irregular, a condition which is in marked contrast with the outline in contact with the yolk. Examination of this latter outline, in unstained sections, suggests that the immediate yolk is being pressed inwards. It is against this bow-shaped compression that the greater part of the glycogen lying in the area is laid down.

In addition, there is usually in such an area an open network of material as well as the numerous granular bodies.

The larger of these lie close to the yolk-sac cells. Since the cells which border an area may be smaller than usual, it is occasionally not easy to distinguish granular body from cell on size alone, but they can be differentiated because of their different reactions to various stains.

After the use of Mallory's connective tissue stain the yolk-sac cells are always a deep orange colour, whereas the contents of the areas and the yolk edge are blue as, incidentally, are the dermis and epidermis.

With a combination of eosin and haematoxylin, the yolk-sac cells take both stains, but the granules only the latter.

These distinctions between yolk-sac cells and the contents of the areas are marked even in those cases where the areas are practically surrounded by the cells, as they may be in stage IX.

The general plan presented by these areas, therefore, is that of the larger granules nearest to the yolk-sac cells, smaller granules farther away and finally a line of what appears to be a dense deposit of glycogen abutting on the yolk.

It might be considered, therefore, that the formation of glycogen, in the later stages of development under review, was proceeding from the yolk-sac cells, or at any rate was being activated by them. Certainly there is no parallel case of the laying down of glycogen in yolk lying near to the embryo. In addition, it may be noted that glycogen is contained in all three tissues of the yolk envelope. Deposits in the yolk-sac cells may be heavy, and to a lesser degree it is found in the dermis and the epidermis or at the common boundary of these two layers.

DISCUSSION

The present observations demonstrate that in the early development of salmon, before the gastrula stage, there is glycogen in the blastoderm cells, in the perivitelline fluid and small amounts in the periblast.

There is a parallel to this distribution in both amphibian and avian development. Woerdemann (1933) describes the blastula of Axolotl with its accumulation of glycogen in many of the cells at the animal pole, with small quantities, or none, to be found elsewhere, except in the perivitelline fluid, which latter storage place has also been recorded for the frog (Needham).

Similarly, Jacobson (1938) records considerable amounts of glycogen, in almost every cell of the blastoderm, in the blastula stage of Aves.

There is also loss of glycogen in the invaginating cells of the gastrula stage in all three groups. There may also be similarity in the time or manner in which glycogen is laid down during the formation of the muscular system and the development of the liver.

It has already been described how, in the salmon (stage III), glycogen is present as isolated granules or streaks during the laying down of the myoblasts in anticipation of the formation of striped muscle fibre. There is like anticipation in the frog and the chick.

Konopacki (1924) records that in the frog glycogen occurs as irregular masses or grains in the myomere cells. During the elongation of the myoblasts and formation of myofibrils, glycogen increases and is localized along the fibrils as small grains or irregular masses in non-differentiated sarcoplasm.

In the chick, according to Allen (1919), glycogen is present in the myotomes 56 hr. after fertilization. Jacobson (1938) states of the chick that when the somites appear glycogen occurs only in the dorsomedial part which probably represents the presumptive myotome tissue.

Differences in the disposition and concentration of glycogen in early and later stages of salmon liver have already been noted. These apparently have their parallel in the chick, for Dalton (1937) has recorded that glycogen is first homogeneous and then in later livers more widely distributed and granular. The disposition of liver glycogen in his Fig. 4 (8-day embryo) and Fig. 3 (17-day embryo) is similar to that in stage VI (Pl. 4, fig. 17) and stage XII (Pl. 4, fig. 18) of the salmon respectively.

In spite of such similarities, however, there are wide divergencies in the carbohydrate metabolism, including glycogen, of the three types. Evidence gathered by Needham (1931, 2) shows that in the developing chick glucose is strongly attacked and glycogen left intact, whereas in Amphibia during development while there is only 7% loss in total carbohydrates, 41% of the original glycogen disappears.

Hayes & Hollett (1940) have demonstrated that the developing larva of salmon finishes up with more glucose than when it starts, and they find this to be true also for glycogen, a point that is in agreement with this paper. In the later stages of development glycogen actually increases more rapidly than glucose, so that about 70 days after hatching it is twice the weight per larva.

Nevertheless, so far as the present investigation is concerned, there is no increase of total glycogen in salmon from 44 to 9 days before hatching. There is a certain similarity at this period with the Amphibia, in which, however, glycogen falls from fertilization to hatching, in that the amounts involved in both cases are comparatively small, being 0·08 mg. per egg for the earliest stage in the frog, according to Brachet & Needham (1935), and 0·06 mg. per egg in the salmon.

Hayes & Hollett (1940) have pointed out that the complete cleavage of the amphibian egg, with its dividing up and sharing out of yolk material, has set up an entirely different set of conditions from those to be found in the meroblastic egg of the teleost. They believe one consequence to be a difference in the form of carbohydrate initially present. In the Amphibia it is glycogen, whereas in the salmon it is free glucose which is more diffusible and therefore better suited to the more complicated task of yolk distribution presented by a meroblastic egg.

This does not preclude the presence of extra-embryonic glycogen in the meroblastic egg, however, for chemical estimations and histological preparations have shown it to be present in both the chick and the salmon during development, and it is practically the only source of glycogen in the former at the beginning of the incubation period. Allen (1919) has shown, by staining methods, that this condition is true for the chick from the earliest stages to 10 days after fertilization. It is concentrated in that part of the yolk-sac nearest to the embryo, the embryonic shield, which corresponds most nearly, in development, to the yolk-sac tissue of the salmon.

In addition, both the chick and the salmon have a well-developed yolk-sac circulatory system to assist in the mechanism of yolk absorption. Allen concluded from these conditions in the chick that 'carbohydrates are stored as glycogen on their way from the yolk to the embryonic tissue'.

Concerning the salmon, there are fat globules in the yolk-sac cells as well as glycogen, so that this tissue, which lies between the yolk and the blood system of the yolk-sac, has a storing or regulating function.

Although the storage of glycogen is less concentrated than in liver cells, there may be heavy deposition in or near the yolk-sac cells, and this is accompanied by disturbances in the peripheral part of the original homogeneous yolk.

Further, the presence of concentrated glycogen (and fat) in the myosepta of the developing embryo suggests a connexion between the laying down of this product and the blood system, since connective tissue, in general, receives material from, and gives up waste material to, blood capillaries.

The conclusion, therefore, that as the growing salmon embryo uses up glucose this is replaced by diffusion from the yolk, is based upon an over-simplification of the process that is actually taking place.

Relationship of Liver and Yolk-sac in Salmon

It has already been pointed out that the yolk mass in the Salmonidae is closely linked to the liver by its vascular supply.

This condition is shown clearly by Portmann & Metzner (1929) in their Fig. 1*a*. Blood from the liver traverses the yolk-sac and then runs to the heart. In the trout it is only 40 days after hatching that there is direct venous connexion between liver and heart.

In the development of certain animals, the embryonic liver does not store up glycogen in the early stages, so that this function is taken on by other tissues which may be extra-embryonic. In general, as soon as it is possible for liver cells to store glycogen, this wider accumulation no longer takes place; it has only been a transitory phase.

What, then, is the position in the salmon embryo? According to Hayes & Hollett (1940), small quantities of liver glycogen were estimated upon the 6th day after hatching, although, in the text, the liver is not accepted by them as a storage organ until 30–40 days after hatching. In their discussion (p. 61) these workers draw attention to the sudden appearance of glycogen in the liver some 40 days after hatching.

Neglecting, as they do, their own figures indicating traces of liver glycogen, then the stage in our histochemical series corresponding to its appearance is the last one, stage XII. There is, indeed, a large quantity of glycogen displayed in the liver at this stage, as well as in the muscles, but, in addition, sections and estimations show that glycogen is still present in the yolk-sac cells and in yolk adjacent to them.

Liver glycogen has not been estimated separately in the present investigation, but if the earliest figures given by Hayes & Hollett (1940) in their table have any significance, then this overlapping of the glycogenic functions of liver and yolk-sac may be pushed as far forward as stage VII, in which there is clear histochemical indications of glycogen in both areas. After this stage the amount of glycogen in the yolk-sac actually increases.

It seems possible to take this overlapping even further. Reaction by liver tissue to fixation and staining methods is so characteristic that what is acceptable as liver glycogen in the sections of stage XII is equally acceptable as early as stage V, which occurred about 5 days before hatching.

The functioning of cells in the yolk-sac, therefore, overlaps the storage of glycogen in the liver. Like the cells of the latter organ, they are absorbing food material and, at the same time, being supplied by a blood supply which is venous. In both cases a certain amount of glycogen may be primarily necessary to enable them to carry out food absorption in the presence of small amounts of oxygen.

The yolk-sac cells are formed originally from the germ layers which spread and enclose the yolk. When the latter is absorbed they form part of the true embryo again, and in this sense the yolk is inside the embryo throughout the various stages. While they encircle the yolk these tissues function as a 'supplementary' rather than a 'transitory' liver, linked closely to the true liver by the venous system.

SUMMARY

1. Quantitative estimations and histological methods have been used to determine the presence and distribution of glycogen in fertilized salmon eggs and subsequent stages of development.

2. A check upon the occurrence of glycogen in each sample was obtained by the use of amylase and the presence of glucose as a result of this technique confirmed, in certain large samples, by the formation of phenylglucosazone crystals.

3. Results of estimations agree with the general distribution of glycogen as shown in histological sections. They differ from those of Hayes & Hollett (1940) who, using water extraction, found no glycogen in stages corresponding to stages I and II and only recorded it as doubtfully present in the yolk-sac.

4. In stages I and II glycogen is concentrated in the blastoderm and perivitelline space. Later the main sources are the muscles, liver and yolk-sac envelope.

5. Glycogen is present in embryonic muscle tissue when fibrils are being laid down (stage III). Subsequently it occurs in both sarcoplasm and muscle fibres.

6. Eight days before hatching (stage V) there is strong staining reaction for glycogen in liver cells, and it is present in all later stages. This glycogen is not obtained from engulfed food or from direct absorption of yolk by liver cells.

7. There is histological evidence that yolk is taken up by the yolk-sac blood vessels after absorption by the yolk-sac cells and dermis. This absorption is accompanied by the appearance of glycogen in these cells and in yolk lying adjacent to them.

8. An increase in amounts of glycogen in both embryo and yolk-sac coincides with a rapid absorption of fat which takes place about 20 days after hatching.

9. The presence of glycogen in blastoderm cells, before the gastrula stage, is similar to the condition in developing Aves and Amphibia. This is true, also, for its appearance early in the formation of muscle tissue. Other similarities are the presence of glycogen in the perivitelline fluid of salmon and Amphibia and the manner of its distribution in the liver of early and late stages of the salmon and chick.

10. There is extra-embryonic glycogen present in the meroblastic eggs of both the salmon and chick. It is concentrated in the embryonic shield of the latter which corresponds, in development, to the glycogen-carrying envelope of the salmon

yolk-sac. Accompanying these deposits in both cases is a well-developed circulatory system to assist in the absorption of yolk.

11. The presence of glycogen in the yolk-sac cells of the salmon refutes a suggestion that the embryo receives glucose only by direct diffusion from the yolk.

12. The liver cells and yolk-sac cells overlap in the function of laying down glycogen during development. The latter, therefore, do not form a 'transitory' but rather function as a 'supplementary' liver, connected with the liver by a venous system until the cells rejoin the true embryo after complete yolk exhaustion.

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EXPLANATION OF PLATES

The distribution of glycogen is shown by black dots and masses. All figures are drawn from sagittal sections. Fixatives and stains listed were used for the particular section represented in the figure. They were not the only fixatives and stains used in each stage.

The number of days before or after hatching is given for each stage. The intervals differ from those in Text-fig. 1, in which the stages have been fitted into the different developmental rate of samples from a later Season.

Abbreviations

BL.	Blastoderm
B.V.	Blood vessel
CN.	Chorion or egg membrane
D.	Dermis
EP.	Epidermis
G.	Glycogen
G.YK.	Open-texture yolk
LV.	Liver
MB.	Myoblast
M.F.	Muscle fibre
M.S.	Myotome
MY.	Myoseptum
N.	Notochord
O.G.	Fat globule
PB.	Periblast
P.M.	Perivitelline membrane
P.S.	Perivitelline space
S.C.	Subgerminal cavity
SP.	Sarcoplasm
SYN.	Syncytium
Y.CH.	Yolk channels or inlets
YK.	Yolk
YS.C.	Yolk-sac cells
YS.E.	Yolk-sac envelope

PLATE 2

Figures drawn from low-power projection through A.A. objective. Final Magnification × 8·8.

Fig. 1. Stage I. 33 days before hatching. Glycogen in blastoderm, periblast and perivitelline space. Fixative: picro-dioxan; stain: Lugol's solution.

Fig. 2. Stage II. 26 days before hatching. Glycogen in blastoderm, periblast and perivitelline space. Fixative: Bouin-Allen; stain: Best's carmine.

Fig. 3. Stage IV. 12 days before hatching. Chorion has been removed. Glycogen in muscle tissue, yolk-sac envelope and small amounts in adjacent yolk. Fixative: picro-dioxan; stain: Best's carmine.

Fig. 4. Stage VI (see also Pl. 4, fig. 17). 2 days after hatching. Glycogen in liver and in and near yolk-sac cells. In muscle sarcoplasm; also in muscle fibre. Fixative: picro-dioxan; stain: Lugol's solution.

Fig. 5. Stage VIII. 16 days after hatching. Distribution of glycogen as in fig. 6. Fixative: picro-dioxan; stain: Best's carmine.

Fig. 6. Stage X. 30 days after hatching. Glycogen in liver, muscle fibres, yolk-sac cells and yolk. Fixative: picro-dioxan; stain: Best's carmine.

PLATE 3

Microscope. Eyepiece 3. Objective $\frac{1}{6}$ in. Final Magnification × 270.

Fig. 7. Stage I. Section through blastoderm showing glycogen in perivitelline space and blastoderm cells. Fixative: picro-dioxan; stain: Best's carmine.

Fig. 8. Stage III. 19 days before hatching. Sagittal section through yolk and yolk-envelope, showing yolk-sac cells, epidermis and chorion. Fixative: picro-dioxan; stain: Lugol's solution.

Fig. 9. Stage V (see also Pl. 4, fig. 15). 5 days before hatching. Section through the yolk and yolk-envelope, showing yolk-sac cells, dermis, epidermis and chorion. Fixative: picro-dioxan; stain: Bauer's.

Fig. 10. Stage VII. 9 days after hatching. Section through yolk and yolk-sac envelope, showing yolk with inlets or rifts, yolk-sac cells, dermis and epidermis. Fixative: picro-dioxan; stain: Best's carmine.

Fig. 11. Stage IX. 25 days after hatching. Section through yolk and yolk-sac envelope. Fixative: picro-dioxan; stain: Bauer's.

Fig. 12. Stage XI. 37 days after hatching. Sagittal section through posterior end of yolk-sac, showing yolk, yolk-sac cells and dermis. Fixative: picro-dioxan; stain: Best's carmine.

PLATE 4

Fig. 13. Stage XII (see also figs. 16, 18). 43 days after hatching. Section showing glycogen in muscle fibres, liver, yolk-sac cells and adjacent yolk. Fixative: picro-dioxan; stain: Best's carmine. Projection through A.A. objective. Final Magnification × 8·8.

Fig. 14. Stage III. Muscle tissue showing glycogen in myoblast syncytium. Fixative: picro-dioxan; stain: Lugol's solution. Eyepiece 3. Objective $\frac{1}{6}$ in. Final Magnification × 270.

Fig. 15. Stage V. Dorsal muscle fibres with glycogen in sarcoplasm, muscle fibres and myosepta. Fixative: picro-dioxan; stain: Lugol's solution. Eyepiece 3. Objective $\frac{1}{6}$ in. Final Magnification × 270.

Fig. 16. Stage XII. Dorsal muscle fibres with glycogen in muscle fibres; also in sarcoplasm and myoseptum. Fixative: picro-dioxan; stain: Lugol's solution. Eyepiece 3. Objective $\frac{1}{12}$ in. Final Magnification × 550.

Fig. 17. Stage VI. Sagittal section of liver. Fixative: picro-dioxan; stain: Best's carmine. Eyepiece 3. Objective $\frac{2}{3}$ in. Final Magnification × 57.

Fig. 18. Stage XII. Sagittal section of liver Fixative: picro-dioxan; stain: Best's carmine. Eyepiece 3. Objective $\frac{2}{3}$ in. Final Magnification × 57.

INVESTIGATIONS ON THE REGIONAL DETERMINATION OF THE CENTRAL NERVOUS SYSTEM

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(With Seventeen Text-figures)

INTRODUCTION

Among the experiments to elucidate the origin of the germ cells in the Urodeles (Nieuwkoop, 1946), one experimental series was investigated, in which the dorsal blastoporal lip of one embryo was transplanted to the presumptive ventral lip of another (cf. Spemann, 1918, 1931*a*).

In order to investigate the development of the germ cells, experimental animals must be reared to an age at which the germ cells are localized in the germ ridges, and show definite differentiation; in the present case, therefore, they have been allowed to develop until they reached an age of about 4 weeks, and all their yolk had been absorbed, which corresponds to about stage 43 (Glaesner). At this stage all the tissues showed complete differentiation. Most of the embryos could not be reared longer, as they could not take food in consequence of abnormalities in head and trunk (mouth, intestinal tube and anus).

In various experiments by other authors, almost all preparations have been fixed at a very early developmental stage (tail-bud stages) in order to prevent mortality. My own experiments demonstrate that this mortality must be ascribed almost completely to extraneous infection. With an accurate sterile working method (described by Woerdeman, 1930), the mortality can be reduced to nil if the egg material is good.

The primary as well as the secondary embryonic rudiments of these duplicitas showed well-differentiated nervous systems. Their structure, especially that of the secondary rudiments, was very interesting (they varied between a complete nervous system and a small fragment of the spinal cord only). The analysis of the structure of these incomplete nervous systems forms the subject of this paper.

RELATIVE POSITION OF PRIMARY AND SECONDARY EMBRYONIC RUDIMENTS

(*a*) *Method of transplantation*

Early gastrulae of *Triturus taeniatus* were used. The dorsal blastoporal lip was isolated as follows. Two sagittal cuts were made along meridians of the egg, to the right and left sides of the dorsal median line, from about the animal border of the marginal zone up the blastoporal margin. Then a transverse cut was made along the animal border of the marginal zone, and the graft was bent outwards. Attached vegetative blastomeres, recognizable by their light colour, were removed from the inner side, after which the graft was completely isolated by a fourth cut along the blastoporal margin (cf. Fig. 1 *a*). The same operation was performed in the host, but on the opposite, ventral, side of the marginal zone. The graft was placed in the wound field in a normal orientation, viz. with its vegetative border against the vegetative field of the host and its median line coinciding with the ventral meridian of the host (cf. Fig. 1 *b*).

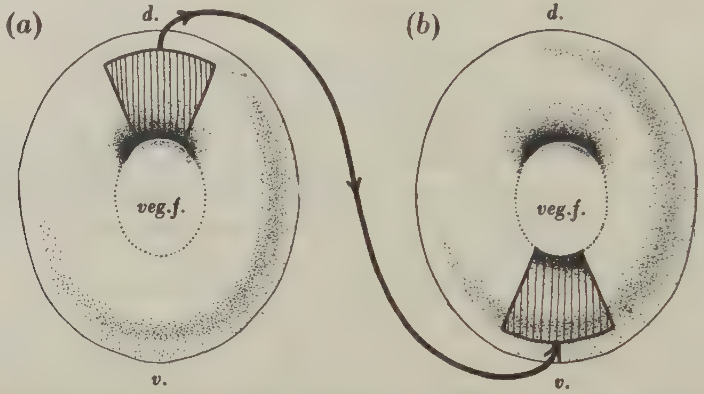

Fig. 1. Diagram of transplantation of dorsal blastoporal lip of early gastrula (*a*) in the place of ventral blastoporal lip of early gastrula (*b*). *d.* dorsal; *v.* ventral; *veg.f.* vegetative field.

(*b*) *Differences in the character of the graft*

An accurate determination of the stage of operation was not of much importance for the original purpose of this transplantation experiment. The operation was, therefore, performed on embryos, ranging from very early (first indication of the dorsal blastoporal lip) to middle (horseshoe-shaped blastopore) gastrula stages. In the first case the graft consisted of presumptive notochord, prechordal plate and perhaps small parts of the cephalic entoderm. In the second case, however, in which external and any already invaginated marginal zone were transplanted together, cephalic entoderm and prechordal plate cell material probably formed no part of the graft. It is therefore clear that the character of the graft varied in individual cases. Unfortunately, individual data are not available.

In a number of cases the graft had been reduced in size by loss of blastomeres, probably owing to damage during the operation. In some cases, therefore, only a small part of the original graft remained, and later invaginated.

(*c*) *Description of the early development of experimental animals**

The first effect of the operation was a marked elongation of the embryo. In comparison with a control which showed a similar deformation after removal of the yolk membrane, elongation was much greater in the experimental animals. These became pear-shaped, the blastopore being situated on the stalk. Two points of invagination were present, giving rise to two archenteron roofs and two neural plates (Fig. 2*a*, *b*). In many cases the secondary neural plate was smaller—shorter and narrower—than the primary one. Both became visible, however, at the same moment. In the embryos examined, I could find only one archenteron, viz. in the primary embryonic rudiment.

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* In this second series in which the embryos are early fixed, *T. alpestris* was used.

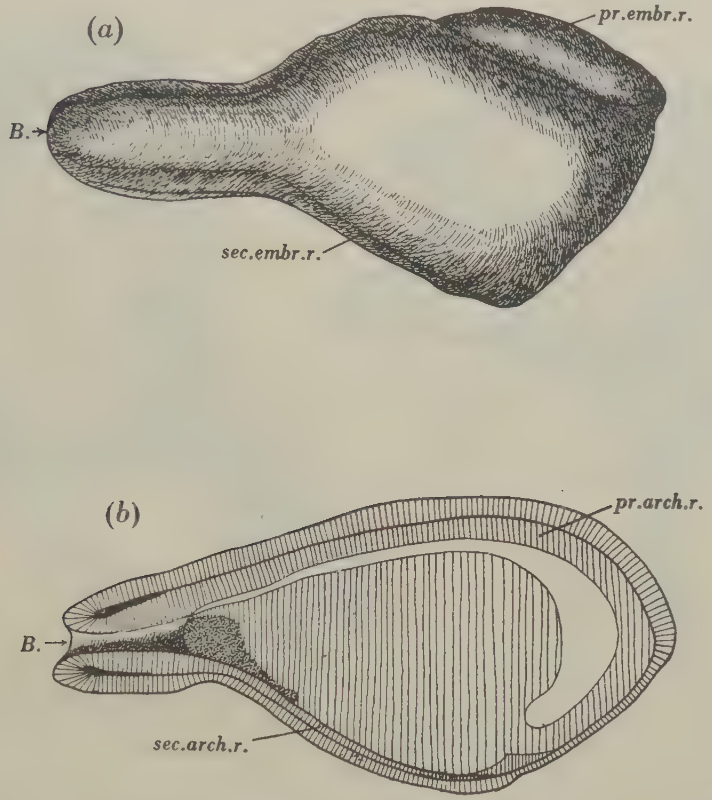

Fig. 2. Duplicitas larva, *T. alpestris* 254 at early neurula stage. (*a*) External view. (*b*) Medial section (left half). Originally most caudal parts of entoderm stained before operation (dark dotted). Enlargement ±40×. *B.* blastopore; *pr.* and *sec.arch.r.* primary and secondary archenteron roof; *pr.* and *sec.embr.r.* primary and secondary embryonic rudiment.

The two embryonic rudiments were situated exactly opposite each other in *T. alpestris* 254 (Fig. 2 *a*, *b*) and 132 (Fig. 3). In Fig. 3 *a*, *b* the notochord and spinal cord of the primary and of the secondary embryo remained separated throughout their lengths. The entoderm mass, on the other hand, was situated chiefly in the anterior part of the trunk. Only a very thin column of entodermal cells was present in the middle trunk region, whereas the caudal part of the trunk was completely free of entoderm. The secondary embryonic rudiment, being shorter than the primary one, was microcephalic. The external form of the duplicitas differed very much from that of controls of the same age. In *T. alpestris* 141 (Fig. 4*a*) the secondary neural plate was much smaller than the primary one. It was situated somewhat to the right of the ventral median line. In the older embryo (Fig. 4*b*) this secondary rudiment had been displaced far to the lateral and even to the dorsal side. The caudal part of its spinal cord was united with the primary cord. The external form of the embryo was, in contradistinction to *T. alpestris* 132 (Fig. 3), scarcely different from a normal embryo of the same age.

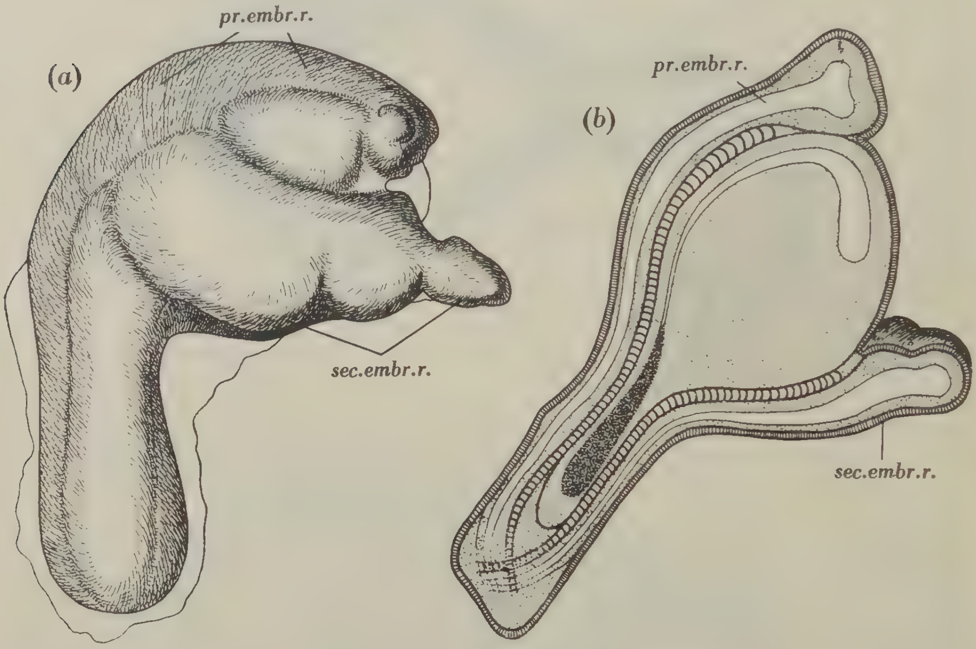

Fig. 3. Duplicitas larva, *T. alpestris* 132 at tail-bud stage. (*a*) External view (embryo attached to cellophane membrane). (*b*) Medial section through primary and secondary rudiments (most caudal entoderm stained as in *T. alpestris* 254); separate primary and secondary notochords, neural tubes and tail-buds. Enlargement ± 30 ×. Abbreviations as in Fig. 2.

(*d*) *General remarks*

The object of all the operations was to obtain two embryonic rudiments, similarly directed and situated exactly opposite each other. The first requirement was fulfilled in all cases, the direction of gastrulation of primary and secondary rudiment being the same and extending from the vegetative to the animal side. In respect to the second requirement the individual cases diverged greatly. It is of interest to consider the causes of this variability.

Sources of errors in the orientation of the graft

(1) In early gastrulae the dorsal median line is difficult to determine. Theoretically, it runs through the middle of the dorsal blastoporal lip. Considerable deviations could be observed, however, in individual eggs.

(2) The graft was intended to consist of the median part of the dorsal blastoporal lip. Small deviations to the right and left could, however, not be avoided.

(3) The wound field was not always exactly opposite the dorsal median line. Here the same errors as in (1) may be expected.

(4) Finally, in the greater number of cases, the orientation of the graft did not entirely coincide with that of the host. The direction of invagination often showed small lateral deviations.

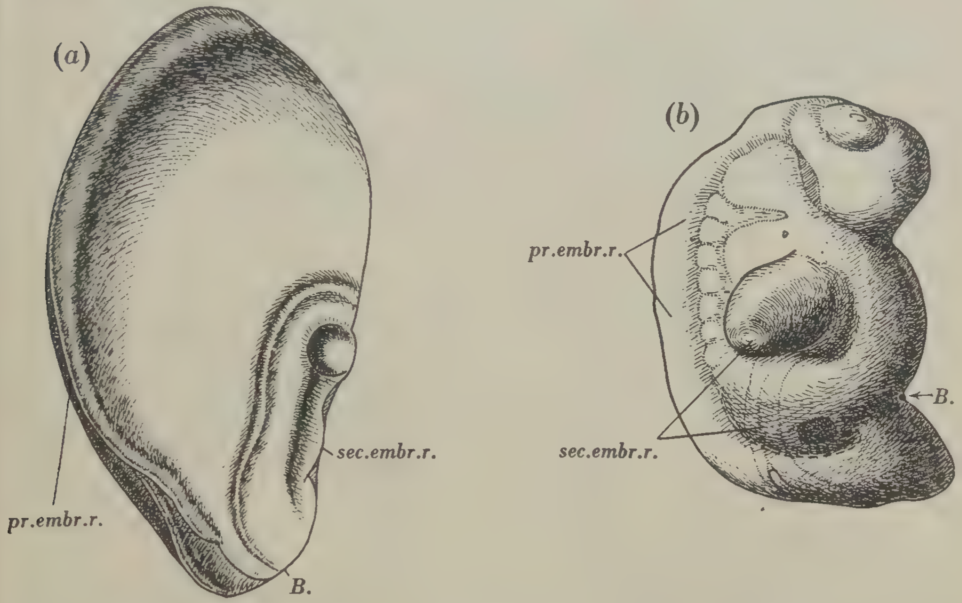

Fig. 4. Duplicitas larva, *T. alpestris* 141. (*a*) External view of primary and secondary neural plates; asymmetrical, lateroventral position of secondary neural plate. (*b*) External view of normal primary and irregular rudimentary secondary rudiment at tail-bud stage; lateral to dorsolateral position of secondary rudiment (most caudal entoderm stained as in *T. alpestris* 254). Enlargement (*a*) ± 35 ×, (*b*) ± 20 ×. Abbreviations as in Fig. 2.

Significance of these deviations in the final position of the graft

The external displacements and those which occur during the first phase of the invagination take place along the meridians (Vogt, 1929*b*; Nieuwkoop, 1946); existing deviations do not increase during this period. After passing the blastoporal lip, however, movements of dorsal convergence arise in the primary and secondary embryonic rudiments. In a normal embryo they extend over the whole of the mesoderm (and ectoderm), except in its medioventral region, where the attractive forces from the right and left sides counterbalance each other. On the left or the right side, the lateral and ventral mesoderm and ectoderm are under the influence of one attractive centre only, viz. the corresponding half of the axial system of the embryo. The greater the distance of a point from the axial system, the weaker is the action of the dorsal median line, and the later its action begins (Vogt, 1929*b*; Yamadà, 1938). The forces of dorsal convergence predominate first in the mesoderm, but later in the ectoderm (elevation and fusion of the neural folds).

In the duplicitas, the lateral mesoderm is under the influence of two opposing attractive centres, and therefore strong attraction arises between the two embryonic rudiments themselves. The latter are, however, separated by the central entoderm mass, and consequently this attraction acts along the right and left sides (cf. the arrows in Fig. 5). With a symmetrical position of the two rudiments, the forces from both sides are equal (cf. Fig. 5*a*). With small lateral deviations from the symmetrical position, however, the forces acting on one side predominate, since the distance between the two centres has been decreased on the side towards the deviation and increased on the other side (cf. Fig. 5*b*). Equilibrium has been disturbed, and the secondary rudiment is displaced towards the primary. This results in a still more marked disturbance of equilibrium. In this way the secondary embryo shifts laterally and, in some cases, even to the dorsal side. This has several consequences, e.g. fusion of the archenteron roofs (notochords) and of the neural plates, especially in the caudal part of the trunk (cf. Fig. 4*a*, *b*).

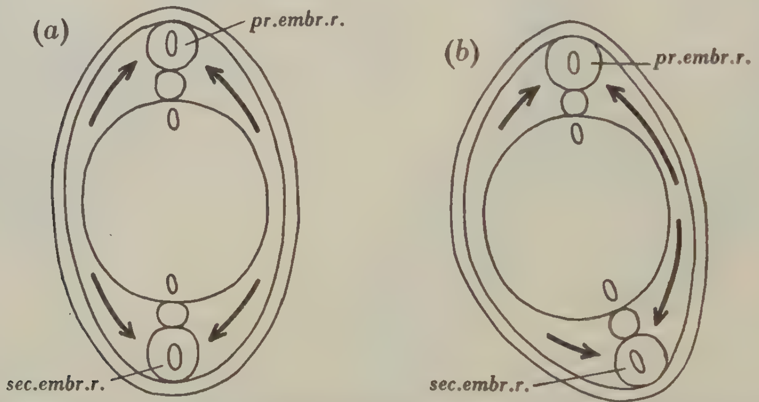

Fig. 5. Diagram of relative positions of primary and secondary rudiment. (*a*) Symmetrical position; equilibrium of dorsal convergence forces of both sides. (*b*) Asymmetrical position; predominance of dorsal convergence forces on side, where both rudiments approach each other. Disturbance of the equilibrium, lateral and even dorsal displacement of secondary rudiment. Abbreviations as in Fig. 2.

The effect of the mutual attraction of the two rudiments on the external form of the duplicitas

The presumptive cephalic mesoderm, first invaginated, is not influenced by the dorsal convergence forces. This cell material reaches its destination before these forces have acted upon it; they appear much later and are, moreover, much weaker in the cephalic region. Therefore, I never observed a mutual attraction of the heads of the two rudiments. They may, for instance, lie almost exactly opposite each other, whereas in the same embryo the trunks are situated entirely on one side of the entoderm mass and the tails have even fused.

The deformation of a normal embryo after removal of the yolk membrane may, probably, be caused by a domination of the posterior displacement of the external marginal zone over the invagination of this cell material. Therefore, the mesoderm accumulates around the blastopore. Not before the end of the gastrulation or the beginning of the neurulation does this cell material begin to be invaginated by the post-gastrulation movements.

In the duplicitas embryos, this accumulation is much more pronounced. The invaginating primary and secondary blastoporal lips may interfere with each other; the ventral extension of the gastrocoele in the head of the primary rudiment may be blocked by the opposite displacement of the secondary archenteron roof, and vice versa. The invagination is, therefore, retarded and continues during almost the whole neurulation period. The appearance of the dorsal convergence movements in the ectoderm greatly accentuates the deformation of the caudal part of the embryo. The neural folds of both neural plates tend to close, thereby exercising a strong pressure upon the underlying mesoderm, which also hinders the invagination. The mesoderm can give way only in a cranial or caudal direction. Therefore, the caudal part of the embryo is considerably lengthened. Moreover, the retardation in the invagination causes a much increased effect of the forces of dorsal convergence. They can act during a much longer time, especially in the caudal part of the trunk. Small deviations of the secondary embryonic rudiment from the symmetrical position are, therefore, markedly augmented during the gastrulation and neurulation periods.

This development has also a strong influence upon the position of the entoderm. Attractive forces in the ecto-mesodermal envelope along both sides of the entoderm mass produce a pressure upon the entoderm in centripetal direction. Under this pressure the entoderm is forced to give way to the cranial or caudal side. The normal gastrulation movements of the invaginating mesoderm having a caudo-cranial direction, the entoderm is most likely to be displaced craniad. (The observation of a secondary expulsion of a part of the entoderm out of the blastopore demonstrates the existence and even the predominance of this centripetal pressure over the normal invagination forces.) These considerations explain the absence of the entoderm in the caudal part of the trunk with a symmetrical position of the two rudiments. Moreover, the absence of the entoderm gives rise to another phenomenon. The two rudiments markedly approach each other, so that their mutual influence (attraction, etc.) increase very much. A direct effect of this situation is the interference with (or even the suppression of) the fusion of the neural folds in the caudal part of the trunk, a condition which I have frequently observed.

(*e*) *Further development*

The foregut cavities enlarged greatly in the cephalic region. Therefore, in some cases the primary and secondary head rudiments were pushed somewhat apart. The splanchnocranium developed normally as far as was possible under these circumstances. In those cases where the two cephalic rudiments had approached each other too much, the splanchnocrania of primary and secondary rudiment fused on their inner sides. In the greater part of the cases I found a duplication of the foregut, whereas the other parts of the intestine were single. This duplication may be caused either by a longitudinal splitting of the archenteron roof or by the implantation of cephalic entoderm.

The following figures give some cross-sections through the trunk of completely differentiated larvae. Fig. 6*a* and *b* show an exactly opposed position of the primary and secondary axial systems. There are two dorsal mesenteries and separate right and left coelomic cavities (Fig. 6*a*). In Fig. 6*b* the intestine has been reduced to a very thin column of entodermal cells surrounded by adjacent myotomes. The two coelomic cavities have disappeared, and the Wolffian ducts of the primary and secondary rudiments have fused in pairs. They lie isolated in the subcutaneous connective tissue and end some sections farther caudad. In the caudal part of the trunk all the cell material of the lateral plates and nephrogenous cords has been transformed into myotomes, probably by the influence of the two notochords. The second duplicitas showed almost exactly opposed heads. In the cranial half of the trunk, however, the two axial systems were already lying asymmetrically. Their planes of symmetry make an obtuse angle with each other (Fig. 7*a*). In the

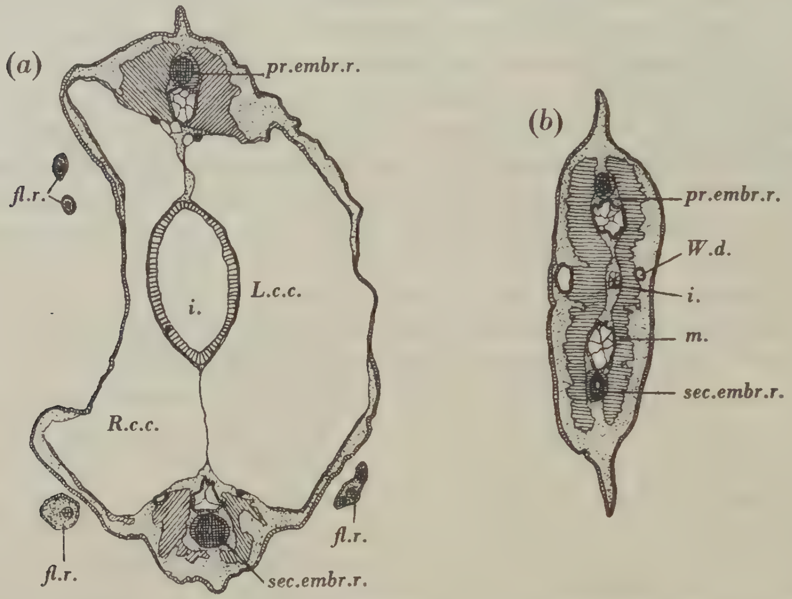

Fig. 6. Cross-sections through trunk of *T. taeniatus* 98. (*a*) Through cranial part of trunk; symmetrical position of primary and secondary rudiments on both sides of central intestinal tube. (*b*) Through middle part of trunk; disappearance of coelomic cavities, fusion of myotomes and Wolffian ducts of primary and secondary rudiment, rudimentary intestinal tube. Enlargement 42 ×. *R.* and *L.c.c.* right and left coelomic cavity; *fl.r.* forelimb rudiment; *i.* intestine; *m.* myotomes; *W.d.* Wolffian duct. Other abbreviations as in Fig. 2.

Legend for Fig. 7.

Fig. 7. Cross-sections through trunk of *T. taeniatus* 102. Enlargement 83 ×. (*a*) Middle trunk, section 129; complete primary and secondary rudiments. (*b*) Middle trunk, section 170; embryonic rudiments approaching each other on left side. (*c*) Middle trunk, section 193; disappearance of coelomic cavity and germ ridges on left side, and fusion of Wolffian ducts and myotomes. (*d*) Caudal trunk, section 230; complete fusion of myotomes and disappearance of fused Wolffian duct on left side; marked mutual approach of both rudiments. *g.r.* germ ridge. Other abbreviations as in Figs. 2 and 6.

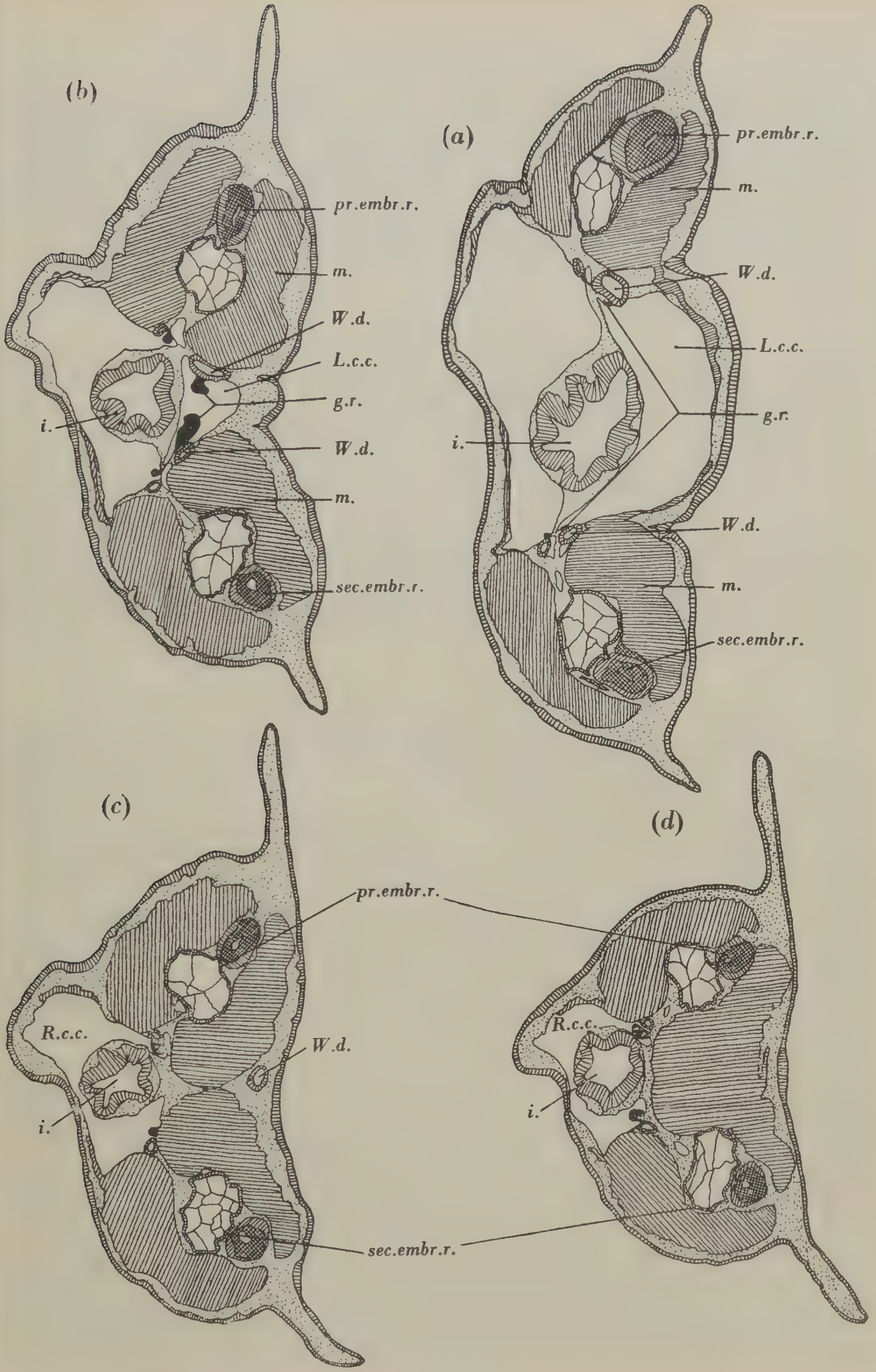
(b)
pr.embr.r.
m.
W.d.
L.c.c.
g.r.
i.
W.d.
m.
sec.embr.r.
(a)
pr.embr.r.
m.
W.d.
L.c.c.
g.r.
i.
W.d.
m.
sec.embr.r.
(c)
(d)
pr.embr.r.
R.c.c.
W.d.
i.
R.c.c.
i.
sec.embr.r.

Fig. 7.

sketches this angle gradually decreases, so that in Fig. 7*d* it is about 90°. Parallel with this displacement of the secondary rudiment, the left coelomic cavity and lateral plate reduce in size and disappear more caudad (Fig. 7*c*). The Wolffian duct (fused) becomes isolated by the fusion of the myotomes. The intestine, however, does not reduce: it hangs down in a normal (here the right) coelomic cavity and communicates normally with the outer world by means of an anal opening.

TECHNIQUE

(*a*) *Sectioning*

Already at an early stage of development the cranial halves of the embryos are strongly divergent, especially the head regions. It was therefore impossible to cut transversely both rudiments in the same section. Moreover, with respect to the investigation on the germ cells, the trunk was the most interesting part, so I tried to cut *the trunk* exactly in cross-sections. This resulted in an oblique sectioning of the heads of the primary and secondary rudiments in most cases. With an exactly medioventral position of the latter, the direction of sectioning varied between transverse and frontal, with only small sagittal deviations. With a lateral position of the secondary rudiment, a still more unfavourable direction of sectioning is obtained, viz. varying between transverse, sagittal and frontal.

(*b*) *Staining method*

The original investigation on the origin of the germ cells necessitated only a general staining method. Therefore, haematoxylin-eosin has been used. An investigation of the nervous system, however, needs much more specific staining methods (e.g. silver impregnation) for making visible the detailed structure of nuclei and nerve tracts. The material allows, therefore, only a general topographic investigation of the brains of primary and secondary rudiments.

Hence this material imposes many restrictions—by unknown variability in the transplantation, lack of data concerning the development, unfavourable direction of sectioning and insufficient specificity of staining method—upon a thorough investigation of the finer structure of the nervous systems induced. The investigation has therefore only a preliminary character.

(*c*) *Method of reconstruction of the brain*

The graphic reconstruction with projection on a sagittal plane was evidently most suitable. In some cases I used also a projection on a frontal plane.

For a graphic reconstruction, two co-ordinates are necessary, viz. in a sagittal projection, a frontal (base) and a vertical median plane, both parallel to the longitudinal axis of the brain. These planes give in every cross-section two mutually perpendicular lines, a horizontal and a vertical. The first requirement for a reconstruction is, therefore, the possibility of an exact determination of the position of the point of intersection of these two lines in every section. Strictly speaking there is no suitable *straight horizontal* line in the brain and head that can be used as a base for the reconstruction. In practice one can, however, consider the median line through the roof of the mouth cavity as a horizontal line, at least in the cranial part of the head. In the caudal part of the brain—the greater part of the secondary rudiments consists only of rhomb- and mesencephalon—the centre of the notochord meets this requirement better. Of course the roof of the mouth cavity does not form a really flat plane; if we still consider this roof as flat, there will be in several cases local deformations in the reconstructions (cf. p. 163, Fig. 12 *a*).

This method of reconstruction (projection on a sagittal plane) can only be used if the median line of the brain as well as the middle of the roof of the mouth cavity can be determined in every section. This is impossible with a sagittal or frontal direction of sectioning. In frontal sections one can only determine the median line of the brain; therefore, one must take a plane other than the roof of the mouth cavity as base plane. Here one can only use the median line of the anterior extremity of the head as a base-line. One has, however, to determine the form of this line separately, as it is not straight, but, on the contrary, strongly curved. This can, for instance, be done with respect to the base-line of the primary rudiment. In an *exact* sagittal series of sections, the median section is present as such. On the contrary, with an *approximately* sagittal direction of sectioning, both the above-mentioned lines fail.

Character and size of the deformations in these methods of projection

(*a*) With an *exactly transverse* direction of sectioning, one has to do only with *deviations of the median line through the roof of the mouth cavity from a straight line.* In most cases these become visible, either in pronounced ventral or dorsal displacements in the longitudinal lines of the brain, or in an abnormal shape of distinct parts (elliptic eye-bulbs, etc.). They can easily be eliminated.

With an *inaccurately transverse* direction of sectioning* one gets important deformations in the reconstruction†, as the plane of sectioning deviates from the ideal plane perpendicular to the longitudinal axis of the brain. One can distinguish (1) deviations in frontal direction (frontal deviations), (2) deviations in sagittal direction (sagittal deviations), (3) deviations in both directions. All these deviations cause deformations of the shape of the brain in the reconstruction. With frontal deviation, parts which are cut in the same section and put vertically above each other in the reconstruction† would be situated in different cross-sections in case of an exactly transverse direction of sectioning. With sagittal deviations, parts situated to the right and left of the median plane appear in one and the same section and, therefore, also on the same cranio-caudal level of the reconstruction, but in reality they are situated at different levels. Only the exact median parts of the brain do not undergo this latter deformation.

Moreover, in all these cases another important deformation arises, viz. a cranio-caudal shortening of the whole reconstruction. With an oblique direction of sectioning the distance between the lines where two successive sections cut the horizontal plane, measured along the longitudinal axis, becomes greater than the real thickness of the sections (cf. Fig. 8 *a*, *b*). The length of a distinct part is, therefore, divided into a smaller number of sections than with a direction of sectioning perpendicular to the longitudinal axis. In the uncorrected reconstruction, the distance between the above-mentioned lines of intersection is always taken as the same. It is clear that the more oblique the direction of sectioning, the shorter the reconstructed brain will be.

These sources of errors are much more difficult to eliminate. First, it is necessary to determine the magnitude of these deviations. The *frontal* deviation may be determined as follows: One measures in the reconstruction the angle between the vertical and a line connecting points *normally* situated vertically one above the other (or in some other well-defined position relative to one another). For this purpose I always used: (1) the line bisecting the angle subtended at the centre of the commissura anterior (ventrally) by the commissura habenularis and the commissura posterior (dorsally) (cf. Fig. 9*c*). This line forms a right angle with the longitudinal axis of the brain in the controls;* (2) the line of junction between the anterior border of the commissura postoptica (ventrally) and the commissura cerebellaris (dorsally). This line makes an angle of about 45° with the longitudinal axis in normal embryos* (cf. Fig. 9*c*). From the values determined in this way one can calculate goniometrically the real deviation.† The *sagittal* deviations can be calculated goniometrically from the angle of deviation made by the lines of junction between exactly symmetrical points in the nervous system with a line perpendicular to the longitudinal axis in frontal projection. Here I always used the most anterior and posterior points of eye-bulb and ear vesicle.

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* I still speak of a 'transverse' direction of sectioning as long as the median plane and the horizontal (base) plane (the roof of the mouth cavity) are cut in every section.

† In the uncorrected reconstruction the two co-ordinates are taken perpendicularly one to the other.

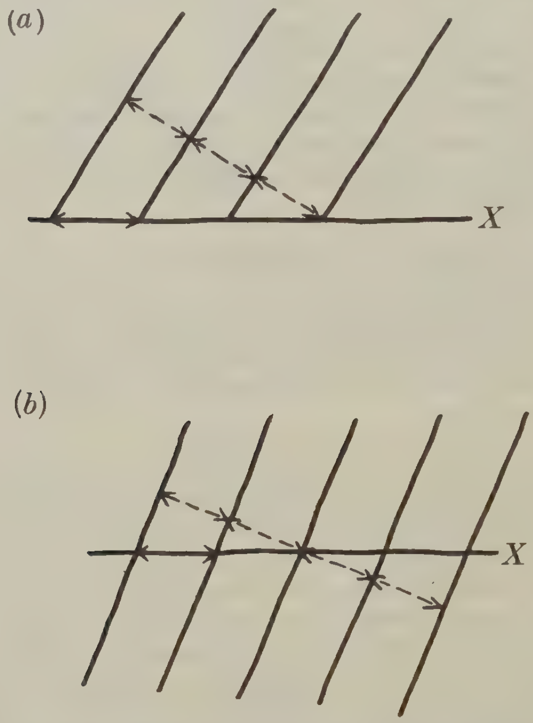

Fig. 8. Diagram of transverse sectioning with frontal (*a*) and sagittal (*b*) deviations. Dark line *X*: horizontal line; other dark lines: successive planes of sectioning; interrupted line: real thickness of sections.

With a planimetric reconstruction method, based on the *real* angles of deformation, one can eliminate the above-mentioned malformations, and one obtains a fairly normal reconstruction‡ of the medial section of the brain. Only the cranio-caudal displacements

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* It has been assumed that these angles have the same values in every control and experimental animal. This evidently holds only within certain limits.

† This calculation has been omitted here, as it has not been applied in the following reconstructions, which give only approximate values.

‡ In the following reconstructions I took into account only the directly determined and not the calculated values, so that these are only approximate.

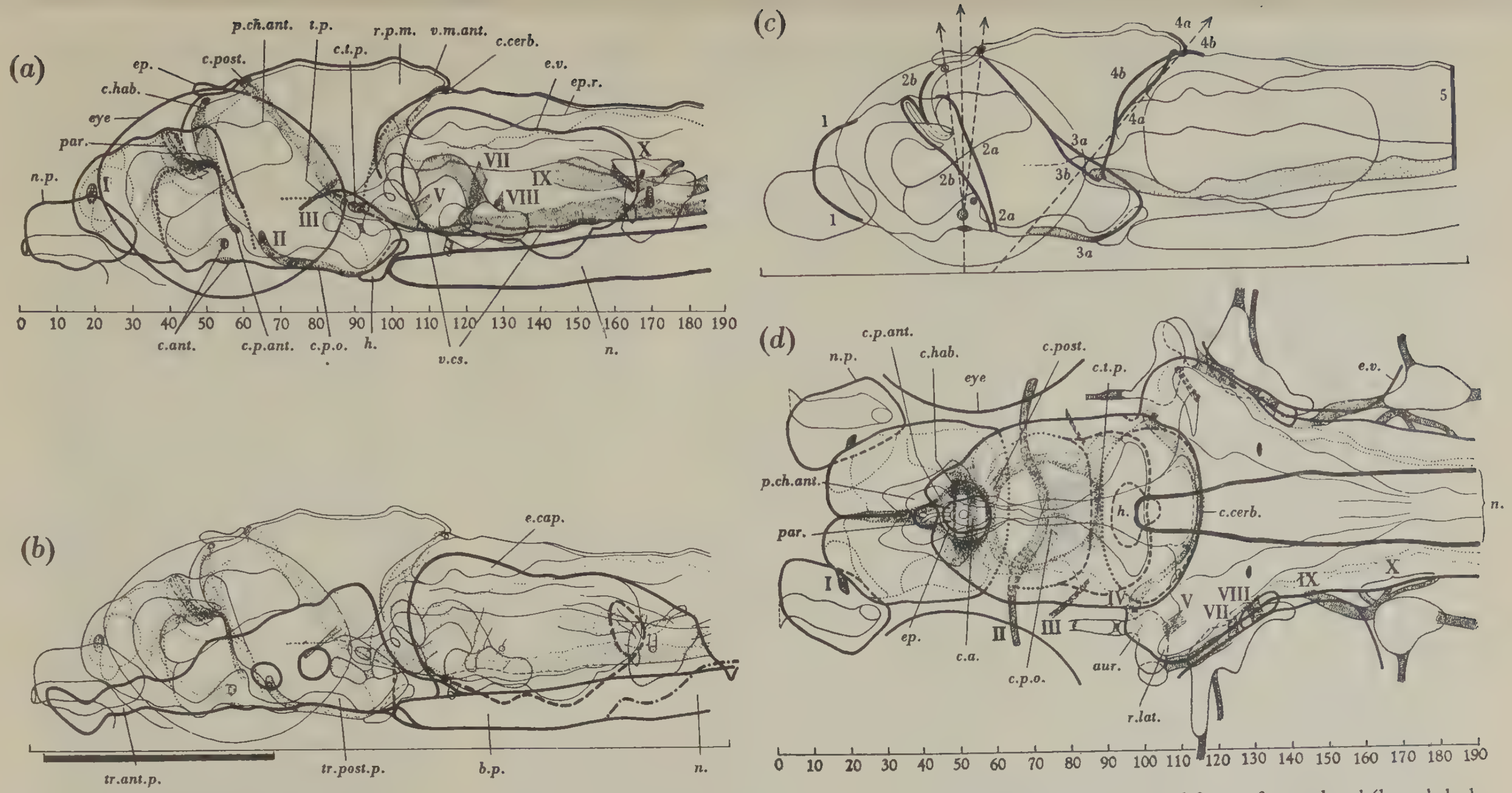

Fig. 9. Graphic reconstruction of brain of control animal, *T. taeniatus* T.o.A. IX. (*a*) Projection on sagittal plane of external form of notochord (broad dark line), brain and sense organs (dark line); of external fissurae (dark dotted line); of internal form of ventricles and cavity of nasal pit (fine line); of boundaries of areas of nuclear tissue (dotted line); of cranial nerves (dotted) and ganglia (continuous and dotted line); and of the course of commissure tracts (wide dotted). Roman numerals: numbers of cranial nerves. *C.a.* commissura anterior; *c.cerb.* commissura cerebellaris; *c.hab.* commissura habenularis; *c.p.ant.* commissura pallii anterior; *c.p.o.* commissura postoptica; *c.post.* commissura posterior; *c.t.p.* commissura tuberculum posterior; *ep.* epiphysis; *ep.r.* epithelial roof of myelencephalon; *e.v.* ear vesicle; *h.* hypophysis; *n.* notochord; *n.p.* nasal pit; *par.* paraphysis; *p.ch.ant.* plexus chorioideus anterior; *r.p.m.* recessus posterior mesencephali; *t.p.* tuberculum posterior; *v.cs.* ventral commissures; *v.m.ant.* velum medullare anterius. (*b*) Sagittal projection of neurocranium. *B.p.* basal plate; *e.cap.* ear capsule; *tr.ant.(post)p.* trabeculae cranii anterior (posterior) part. (*c*) Lines used as boundaries between successive brain parts (cf. text on p. 158). (*d*) Frontal projection of external form of brain and sense organs, internal form of ventricles and course of commissures. (Ventral commissures of met- and myelencephalon have been omitted.) *Aur.* auricle; *r.lat.* recessus lateralis. Enlargement 62·5 ×.

of lateral parts (eyes, ear vesicles, auricles of the rhombencephalon, etc.) are difficult to eliminate in these sagittal reconstructions.

(*b*) With a *frontal* direction of sectioning, similar deviations, now in sagittal and transverse direction, play a part. They can be calculated similarly.

(*c*) With a *sagittal* direction of sectioning we need an exact determination of the position of the successive sections with respect to a *horizontal transverse* line. Suitable transverse lines are, however, lacking in brain and head. The roof of the mouth cavity forms in mediolateral direction a curved plane. At the most the horseshoe-shaped commissura cerebellaris may be used. With a marked deviation from the sagittal direction of sectioning, all these points of reference become unreliable, even for getting approximate values.

The unfavourable direction of sectioning causes many difficulties, often insuperable, in reconstructing the nervous systems of the experimental animals. This last restriction holds good especially for the quantitative determination of the sizes of the separate parts of the brain from the graphic reconstructions.

This determination of size has been carried out as follows: The areas of the brain parts (tel-, di-, mes- and rhombencephalon) and of the sense organs (nasal pit, eye and ear vesicle) were determined in the sagittal projection with the aid of a planimeter. I used the following borders of the successive brain parts: anterior border of the brain (1), lateral margin of the fissura transversa cerebri (anterior) (2*a*), medial border of the fissura transversa cerebri and cranial border of the corpora habenulae (2*b*), anterior border of the commissura posterior and caudal border of the infundibulum (3*a*), anterior border of the commissura posterior and commissura tuberculum posterior (3*b*), the commissura cerebellaris and commissura ventralis (4*a*), auricle of the rhombencephalon and commissura ventralis (4*b*), and the vertical through the ventral root of the first spinal nerve (5) (telencephalon, lines 1 and 2*a*; diencephalon, lines 2*b* and 3*a*; mesencephalon, lines 3*b* and 4*a*; and rhombencephalon, lines 4*b* and 5; cf. Fig. 9*c*).

DESCRIPTION OF THE BRAINS OF A CONTROL AND OF THE PRIMARY AND SECONDARY RUDIMENTS OF THE EXPERIMENTAL ANIMALS

The whole series consists of thirty animals, some of which were, however, unsuited for further investigation in consequence of injury during the preservation (one animal) or very unfavourable direction of sectioning (two animals).

Description of a control

Fig. 9*a* and *d* shows the course of the commissures and nerves of the control, *T. taeniatus* T.o.A. IX, in sagittal and frontal projections.

In the telencephalon (about section 15–60), we observe one pair of nerves, the nervi olfactorii I (20), running to the nasal pits, and two commissures, the commissura anterior and the commissura pallii anterior, situated in the torus transversus. The first consists of two parts, a round part and a flat ventral part. The commissura anterior forms the connexion between the two basal regions, and the commissura pallii anterior between the two mediodorsal primordia hippocampi (cf. Fig. 9*a*, *d*). The telencephalon contains a large ventriculus impar and only small ventriculi laterale. Moreover, the unpaired ventricle is partly filled up by the plexus chorioideus anterior. From the diencephalon, extending dorsally from about section 45–60 and ventrally from 60–100, arises one pair of nerves, the nervi optici II (70), running to the large eyes. Here we meet a ventral commissure, the commissura postoptica bordered anteriorly by the chiasma opticum and the recessus opticus, and a dorsal commissure, the commissura habenularis. The first forms the connexion between the two thalami optici, the second between the corpora habenulae. Other characteristic parts of the roof of the diencephalon are the paraphysis (40–50), the epiphysis (50–60) and the plexus chorioideus anterior in the third ventricle. In the bottom we meet the infundibulum with hypophysis extending far caudad, the latter bordering the tip of the notochord. The mesencephalon has a triangular form; the sides of the triangle are formed by the commissura posterior, commissura cerebellaris and the tectum opticum. The most caudal part of the mesencephalon ventricle is formed by the dorsal recessus posterior mesencephali which underlies the velum medullare anterius. Ventrally, the mesencephalon is very narrow and consists chiefly of the tuberculum posterior and the commissure of the same name (85–95). The nervus oculomotorius III (ventrolaterally 85) and the nervus trochlearis IV (dorsolaterally 100) originate from this brain part. Finally, the rhombencephalon, the greatest brain part, extends from 95 to about 190 and is bordered anteriorly by the fissura isthmi and the two lateral auricles, in which the fourth ventricle forms the right and left recessus lateralis. From this part of the brain many nerves arise, the most ventral and cranial of which is the nervus trigeminus V (110), to which belongs a first group of ganglia. The nervus facialis VII (125) originates somewhat dorsocranially to the nervus acusticus VIII (130); both communicate with a second group of ganglia. The eighth nerve innervates the ear vesicle. The nervus glossopharyngeus IX (145) joins with the various roots of the nervus vagus X (160–180); to both nerves together belongs a third group of ganglia. One of the most characteristic features in the rhombencephalon is formed by the Mauthner's cells (about 130) belonging to the vestibularis system.

Fig. 9*b* shows the form and position of the neurocranium, the cranial half of which is formed by the two trabeculae cranii. Their anterior part bears many teeth, while the posterior part shows two apertures for the passage of the second and third nerves, respectively. The caudal half of the neurocranium consists of the basal plate, in which the notochord is embedded, and of both ear capsules, surrounding the ear vesicles.

Description of the experimental animals, arranged in a series with gradually increasing reduction of the nervous system

(1) *T. taeniatus* 97/99. The primary as well as the secondary rudiment show an entirely complete structure with normally proportioned brain (cf. Fig. 10*a*, *b*). The neurocranium has also differentiated normally.

(2) *T. taeniatus* 149/8 and (3) *T. taeniatus* 158/9 give the same picture as the first animal. They do not show any abnormality.

(4) *T. taeniatus* 164. The primary rudiment has synophthalmic eyes and a single, median nasal pit (monorhinic). The caudal part of the brain (mes- and rhombencephalon) is normal, whereas di- and telencephalon show abnormalities. In the hypothalamic region the infundibulum and hypophysis are lacking. The tuberculum posterior fails. The commissura postoptica is separated from the commissura tuberculum posterior by a narrow band of nuclear tissue only. The nervus opticus has lost, probably secondarily, its communication with the brain. All parts of the diencephalon, with the exception of the infundibulum, the hypophysis and the synophthalmic eyes, have been normally formed. The telencephalon shows only an indication of a paired structure (two nervi olfactorii, running to the single nasal pit, lateral extension of the unpaired ventricle). The telencephalon is, moreover, markedly reduced, but it contains the two commissures, commissura anterior and commissura pallii anterior. Parallel with the abnormal structure of brain and sense organs, the mouth cavity and the neurocranium have been deformed markedly. There is only one median trabecula, extending craniad up to the caudal border of the telencephalon. Its cranial part does not bear teeth; these are situated more caudally against

the posterior part of the trabecula and form only one tooth field in the roof of the mouth cavity. This roof is markedly curved in a ventrocranial direction. The secondary rudiment has a strongly aberrant form, in consequence of a dorsal crack in the rhombencephalon, an incomplete development of infundibulum and hypophysis and the presence of only one (right) telencephalon hemisphere. There has been formed only one (right) nasal pit and one eye (the right eye) displaced to the left side. Finally, the ear vesicles have developed

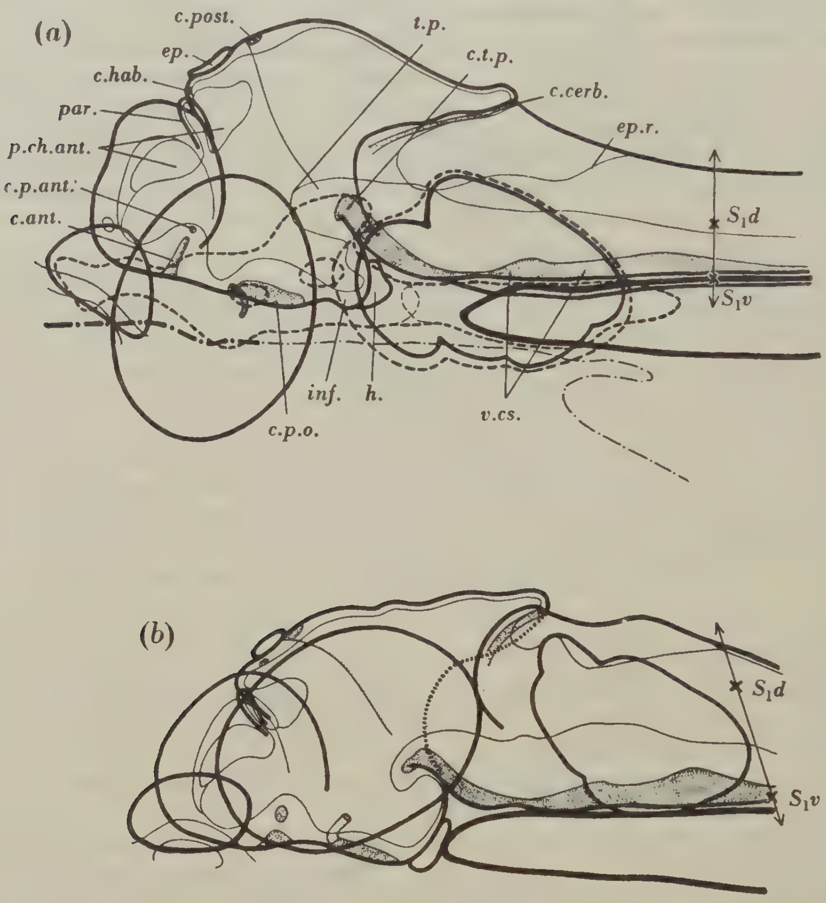

Fig. 10. Similar reconstruction of duplicitas, *T. taeniatus* 97/99. (*a*) Secondary rudiment, (*b*) primary rudiment. External form of notochord: broad dark line; external form of brain and sense organs: dark line; boundary of right auricle: dark dotted line; course of ventricles and cavity of nasal pit: fine line; course of commissures: dotted line; external form of neurocranium: interrupted line; dorsal extremity of mouth cavity and esophagus: interrupted, dotted line (broad: position of tooth field). S_1v (d), ventral (dorsal) root of first spinal ganglion. Other abbreviations as in Fig. 9. Enlargement 83 ×.

unequally, viz. a large right and a small left. In size the parts of the brain are nearly equal to the corresponding parts of a normal rudiment (cf. Table 2). Except for the absence of one eye, the brain is normal from the diencephalon backwards, whereas only one-half of the telencephalon has been formed.

(5) *T. taeniatus* 113/5. The primary rudiment is complete and normally proportioned. In the secondary rudiment (Fig. 11*a*) one observes practically the same deviations from

the normal structure as in the primary rudiment of *T. taeniatus* 164. Here there are two small eyes situated ventrally to the brain and connected with it by one nervus opticus which splits at some distance from the brain. Moreover, there is only one rudimentary nasal pit with two small nervi olfactorii situated close together. The diencephalon lacks an infundibulum and hypophysis; there is, in fact, no trace of it, so that the commissura postoptica

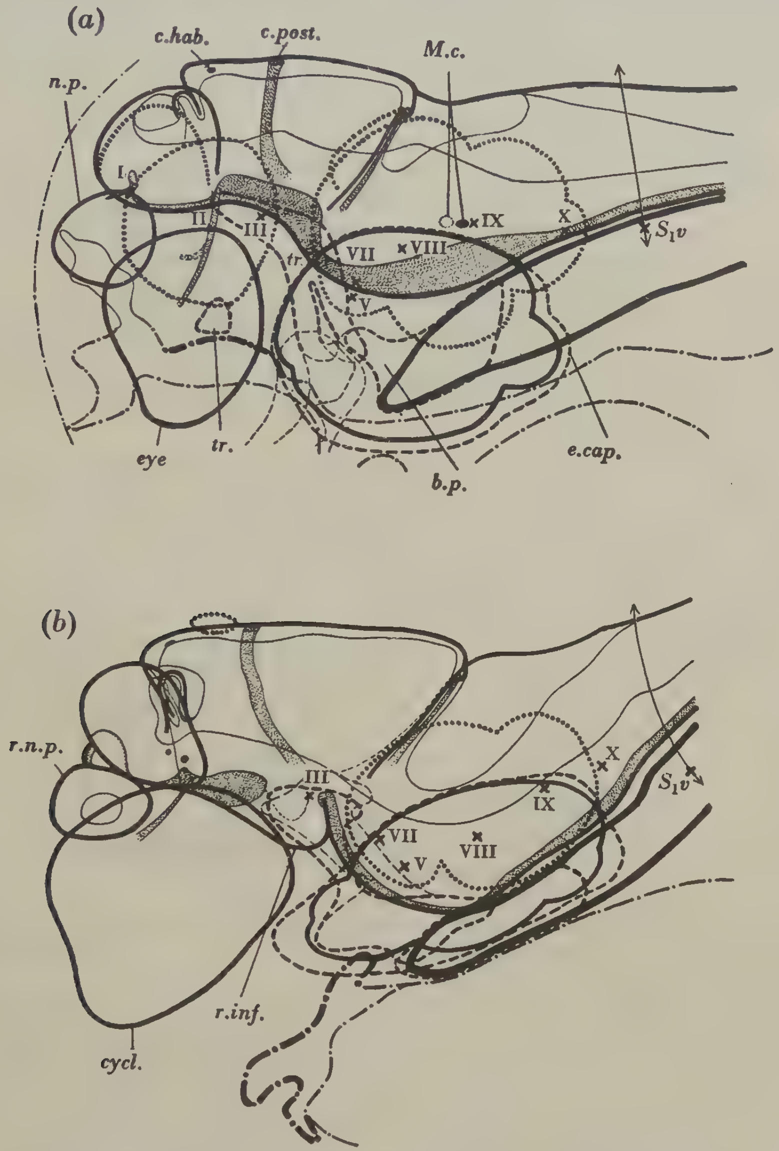

Fig. 11. (*a*) Reconstruction of brain of secondary rudiment of *T. taeniatus* 113/5. Very irregular neurocranium and form of mouth cavity; continual band of ventral commissures. Dark dotted lines: external boundaries of right hemisphere, eye and ear vesicle; ×, origin of cephalic nerves; *M.c.* Mauthner's cells. (*b*) Similar reconstruction of brain of secondary rudiment of *T. taeniatus* 92/4. *r.n.p.* rudimentary nasal pit. *r.inf.* rudimentary infundibulum; *cycl.* cyclopic. Other abbreviations as in Figs. 9 and 10. Enlargement 83 ×.

borders on the commissura tuberculum posterior. The tuberculum posterior itself also fails. The recessus opticus being absent, the commissura postoptica also borders on the commissura anterior of the telencephalon. There is, therefore, a continual band of commissures along the whole ventral side of the brain. The paired structure of the telencephalon is only indicated, and this region is markedly reduced in size. The caudal parts of the brain are completely normal. The neurocranium consists of two ear capsules, basal plate and only one trabecula, the caudal part of which is double. It borders on two cartilages of the splanchnocranium (the palatoquadrata ?). The roof of the mouth cavity contains one tooth field in connexion with a small cartilage, probably belonging to the trabeculae.

(6) *T. taeniatus* 92/4. Only the secondary embryo shows abnormalities. This animal is cyclopic and monorhinic (cf. Fig. 11*b*). One nervus opticus runs to the large eye. The recessus opticus and also the chiasma are absent, so that the commissura anterior borders on the commissura postoptica. There is only a rudimentary infundibulum without ventricle (?). A real tuberculum posterior therefore fails. An hypophysis has not been formed. As in *T. taeniatus* 164 I and 113/5 II all parts of the diencephalon with the exception of infundibulum (rudimentary), hypophysis and cyclopic eye are normally proportioned. The telencephalon is unpaired, but contains two commissures. The single rudimentary nasal pit is innervated by two small nervi olfactorii, lying close together. The forebrain is bent dorsad and diverges from the mouth cavity. The trabeculae (two in number) are strongly curved and end on both sides of the infundibulum. The tooth field borders on the caudal parts of the trabeculae.

(7) *T. taeniatus* 156/5. Both embryos (Fig. 12*a*, *b*) contain rather small brains, much less developed than, for instance, the brains in *T. taeniatus* 97/99. The plexus chorioideus anterior has not yet been formed in the normally proportioned primary brain. The sense organs are smaller than in embryo 97/99. The secondary rudiment (Fig. 12*a*) shows a rudimentary telencephalon with a similar nasal pit. The commissura anterior borders on the commissura postoptica, as the recessus opticus is lacking. There is only one eye (right). The diencephalon has formed a large infundibulum, but without a hypophysis. All the other parts of the brain are normal. The neurocranium has differentiated only in part. The trabeculae consist of some small cartilages lying ventrally to the infundibulum. The mouth cavity is a ventral diverticulum of the foregut of the primary embryo; it contains no teeth. A cartilage of the splanchnocranium extends to the tip of the basal plate.

(8) *T. taeniatus* 148/7 forms almost an image of *T. taeniatus* 156/5. The only differences are: the primary embryo has small plexi in the tel- and diencephalon ventricles; in the secondary embryo no direct connexion between the commissura anterior and postoptica exists. Here only the left eye is present. The mouth cavity contains one tooth field in connexion with a rudimentary basal plate. Trabeculae have not yet differentiated. As in the last animal, all parts of the brain from the diencephalon backwards are completely formed; only the telencephalon has been reduced.

(9) *T. taeniatus* 145/6. The primary embryo is very interesting; the secondary one consists only of a piece of spinal cord and can be omitted here. The primary embryo has a very small telencephalon with a similar small rudimentary nasal pit, whereas all other parts of the brain have been differentiated normally, with the exception of eyes, nervi optici and hypophysis, which are absent. The neurocranium is defective. The trabeculae consist of two separate parts, a caudal part bordering the rhombencephalon and a cranial situated ventrally to the infundibulum. The latter cartilage has induced a tooth field in the roof of the mouth cavity. The cranial part of the notochord is absent; it begins at a level with the middle of the rhombencephalon.

(10) *T. taeniatus* 91/4. The primary embryo cannot be described in detail. It has a strongly abnormal form, as the midbrain has been everted instead of folded in. All the

brain parts are present, however. In the diencephalon, the infundibulum and hypophysis are absent. The secondary embryo is very interesting (cf. Fig. 13*a*). It begins with the diencephalon, not a single trace of the telencephalon being present. The epithalamus has been formed normally; epiphysis, corpora habenulae with commissura habenularis, paraphysis and even an indication of the plexus are found. Their position has, however, been markedly changed by the absence of the telencephalon, but agrees very well with that in Fig. 12*a*, where only a small telencephalon had been formed. An infundibulum and hypophysis fail in the hypothalamic region, but a small commissura postoptica is present.

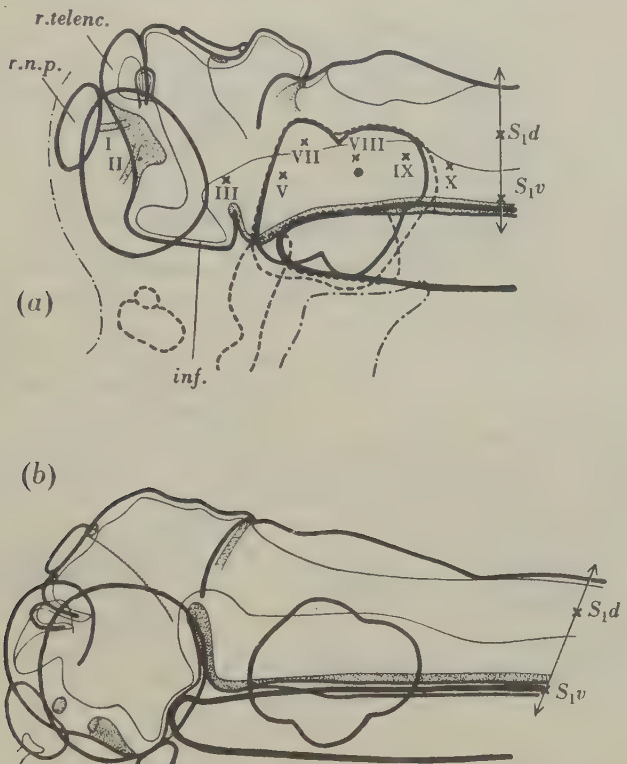

Fig. 12. Reconstruction of brains of primary (*b*) and secondary (*a*) rudiments of *T. taeniatus* 156/5. *R.telenc.* rudimentary telencephalon. Other abbreviations as in Figs. 9 and 10. Enlargement 83 ×.

One nervus opticus running to the single eye (right) originates from the left side. The commissura postoptica nearly borders on the commissura tuberculum posterior of the mesencephalon. A tuberculum posterior fails. The caudal part of the brain shows no abnormality. Again all the brain parts formed are normally developed, only with the exception of infundibulum, hypophysis and eyes. The neurocranium has hardly differentiated and a mouth cavity fails completely.

(11) *T. taeniatus* 154/5. The primary embryo is complete, the secondary one begins with a reduced diencephalon. The epithalamus consists of an epiphysis, lying at the front end of the brain, corpora habenulae with their commissure and a plexus chorioideus, lying in an abnormal place, viz. between the commissura habenularis and commissura posterior. Eyes are lacking. In the hypothalamus the commissura postoptica has been developed and

nearly borders on the commissura tuberculum posterior of the mesencephalon. All parts of the diencephalon with the exception of eyes, infundibulum and hypophysis have been formed, but in a much smaller size than in the normal brain. From the mesencephalon to the spinal cord the brain shows no abnormalities. The neurocranium has hardly differentiated and a mouth cavity fails entirely.

(12) *T. taeniatus* 109/110. The primary rudiment shows no abnormalities. The secondary embryo (Fig. 13*b*) has a normally formed mes- and rhombencephalon. The foremost part is a reduced diencephalon, the following parts of which can be identified: epiphysis, lateral masses of nuclear tissue (thalami optici ?) and commissura postoptica. We have to conclude, therefore, that a partial development and not a reduction of the whole diencephalon, as in the last animal, has taken place here. The neurocranium has hardly differentiated. The foregut of the primary embryo has extended in the caudal direction up to a level beyond the tip of the notochord in the form of a narrow, blind, entodermal tube.

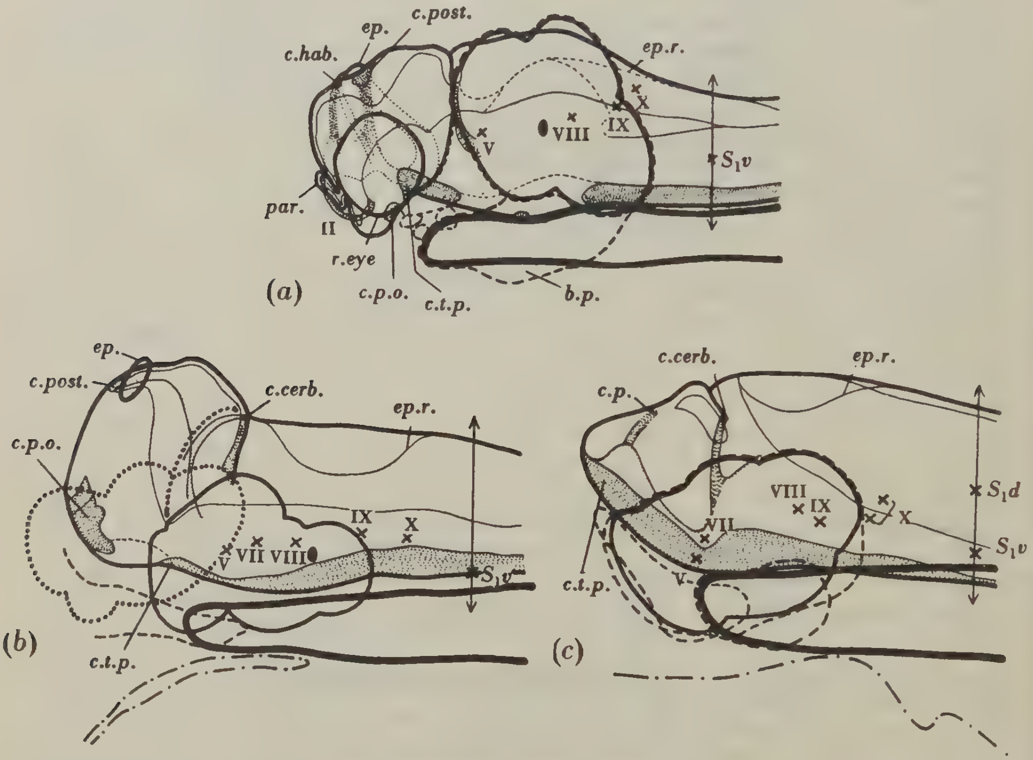

Fig. 13. (*a*) Reconstruction of brain of secondary embryo of *T. taeniatus* 91/4. Complete absence of telencephalon. (*b*) Reconstruction of brain of secondary embryo of *T. taeniatus* 109/110. Dotted lines of right ear vesicle and auricle. (*c*) Reconstruction of brain of secondary embryo of *T. taeniatus* 90. Complete absence of tel- and diencephalon. Abbreviations as in Figs. 9 and 10. Enlargement 83 ×.

(13) *T. taeniatus* 90. The primary embryo shows the same abnormalities as *T. taeniatus* 91/4 I. The secondary brain (Fig. 13*c*) begins with the mesencephalon. It shows an indistinct commissura posterior situated at the front end, a small isolated ventricle, the recessus posterior mesencephali, and a large ventral commissure (here also situated cranially), the commissura tuberculum posterior, forming one whole with the commissures

of the rhombencephalon. The rhombencephalon has a normal structure. All the parts of the brain from the mesencephalon backwards have been formed normally. The neurocranium consists of two ear capsules, basal plate and a single medial, caudal part of the trabeculae.

(14) *T. taeniatus* 102/4. The primary embryo shows no peculiarities. The secondary embryo, however, is difficult to understand. The brain begins with a small oblong part, extending dorsocaudad into the centre of the next part. It must be a part of the mesencephalon, as the next part, the rhombencephalon, begins dorsally with the commissura cerebellaris and ventrally with a continual band of commissures. This mesencephalon cannot be analysed further, its form and differentiation being too aberrant. The rhombencephalon contains a normal fourth ventricle with an epithelial roof. Two normal ear vesicles are situated symmetrically on both sides of the brain. The neurocranium is formed by a small basal plate, two ear capsules and an irregularly curved trabecular cartilage. The primary mouth cavity extends caudad up to a level beyond the tip of the notochord.

(15) *T. taeniatus* 100/101. Only the secondary rudiment demands our attention. The cranial part of the brain has been rotated around its longitudinal axis by about 90°, which greatly hinders the analysis. A commissure originates from the sides of the brain and crosses the dorsal median line just before the beginning of the epithelial roof of the ventricle. Further, the fifth nerve arises somewhat caudally to the front end of the brain, and two normal ear vesicles are situated on both sides of the latter. All these facts demonstrate that we have to do with a complete rhombencephalon. Any trace of a mesencephalon is lacking. The neurocranium has formed basal plate, ear capsules and a median small cartilage, possibly representing the caudal part of the trabeculae. A small oblong cartilage lies to the left side but I cannot identify it.

(16) *T. taeniatus* 96/101. The secondary embryo (Fig. 14*a*) has a regular shape and begins with the rhombencephalon. The commissura cerebellaris crosses the dorsal median line at the front end of the brain. All the nerves of this part of the brain can be identified. The neurocranium consists of the basal plate and two ear capsules surrounding the normal ear vesicles. Cartilages of the splanchnocranium are situated anterior to the front end of the brain.

(17) *T. taeniatus* 150/148, (18) *T. taeniatus* 114/5 and (19) *T. taeniatus* 98/99 (Fig. 14*b*) can be described together. They show the same structure. The brain begins with the fifth nerve, a large band of commissures on the bottom of the brain, and a normal epithelial roof of the ventricle (cf. Fig. 14*b*). These brains consist of a myelencephalon only. Two normal ear vesicles are situated on either side of the brain, and are surrounded by ear capsules. One can further distinguish a basal plate and in the last-mentioned two animals a medially situated cartilage, probably belonging to the splanchnocranium. The foregut of the primary rudiment has formed a long, narrow tube along the ventral side of the notochord.

(20) *T. taeniatus* 106/7, (21) *T. taeniatus* 105/7, (22) *T. taeniatus* 93/4, (23) *T. taeniatus* 163/2, (24) *T. taeniatus* 161/2 and (25) *T. taeniatus* 111/2 also show identical structure of the brain. Here only the caudal half of the myelencephalon has been developed. In all these cases, the brain begins with the eighth nerve or nerves, running to the ear vesicle(s). Mauthner's cells, two in number and present in all these cases, lie at some distance from the tip of the brain. *T. taeniatus* 106/7 shows two lateral ear vesicles and two auditory nerves. The notochord extends up to a point anterior to the brain. *T. taeniatus* 105/7 has two ear vesicles, lying anterior to the brain and bordering on each other in the median plane. The notochord forms a small oblong piece under the tip of the brain only; other traces of it are lacking in the head. The primary embryo contains only one eye and one nervus opticus (right). The left trabecula has an abnormal form and consists of three

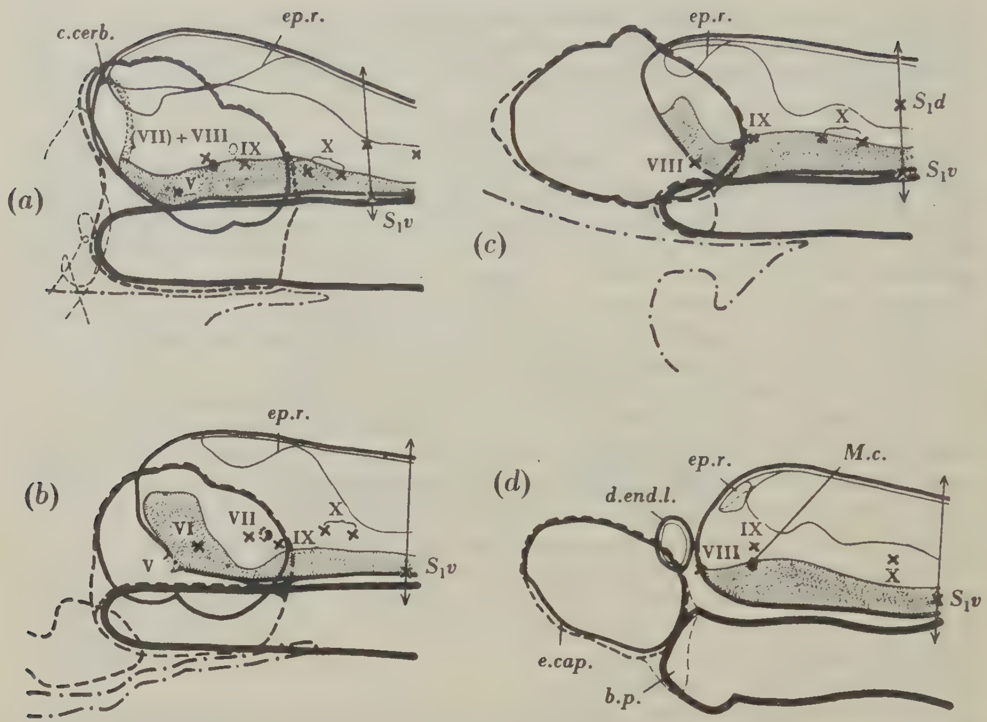

Fig. 14. (*a*) Reconstruction of brain of secondary rudiment of *T. taeniatus* 96/101. Complete absence of tel-, di- and mesencephalon. (*b*) Reconstruction of brain of secondary rudiment of *T. taeniatus* 98/99. Complete absence of tel-, di-, mes- and metencephalon. (*c*) Reconstruction of brain of secondary rudiment of *T. taeniatus* 93/4. Myelencephalon from the eighth nerve; two ear vesicles, bordering each other in median plane. (*d*) Reconstruction of brain of secondary rudiment of *T. taeniatus* 111/2. Myelencephalon from the eighth nerve; one median ear vesicle. *D.end.l.* ductus endolymphaticus. Other abbreviations as in Figs. 9 and 10. Enlargement 83 ×.

isolated parts, the last of which has strongly extended in a dorsal direction. *T. taeniatus* 93/4 (Fig. 14*c*) has also two medially situated ear vesicles, innervated by one nerve. *T. taeniatus* 163/2 shows one large median, bilaterally symmetrical ear vesicle, innervated by two nerves. Here about six Mauthner's cells are present. The notochord extends craniad beneath the whole ear vesicle. *T. taeniatus* 161/2 has formed one median, bilaterally symmetrical ear vesicle of normal size, probably innervated by two nerves. Finally, *T. taeniatus* 111/2 (Fig. 14*d*) shows one median ear vesicle of reduced size and one auditory nerve. The tip of the notochord extends farther craniad than the brain as in the animals 20, 21, 23 and 24. The neurocranium consists of ear capsule(s) and a small basal plate in all these cases, whereas a ventral diverticulum of the foregut of the primary embryo is present only in the animals 20, 21, 22 and 24.

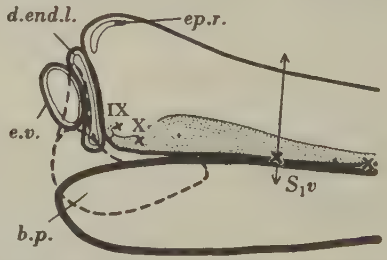

Fig. 15. Reconstruction of brain of secondary rudiment of *T. taeniatus* 103/4. Myelencephalon from the ninth nerve; rudimentary ear vesicle. Abbreviations as in Figs. 9, 10, 11 and 14. Enlargement 83 ×.

(26) *T. taeniatus* 103/4 is a very interesting animal (Fig. 15). While the primary embryo is completely normal, the brain of the secondary is very small and consists of the most caudal part

of the myelencephalon only. It begins with the ninth nerve and shows a very small ventricle with epithelial roof, situated dorsally in the tip of the brain. Mauthner's cells are lacking. A very rudimentary ear vesicle lies in front of the brain, consisting of a very small, oblong ear vesicle and a narrow ductus endolymphaticus. This is also visible in Fig. 14*d*.

Some other experimental animals, *T. taeniatus* 151/2, 160/159 and 108/110, contain only spinal cord inductions; they are therefore omitted here.

DISCUSSION OF EXPERIMENTS

(*a*) *Regulation in the archenteron roof*

I. *Mediolateral regulation*

One of the most remarkable facts is the exactly bilaterally symmetrical structure of the nervous systems induced.* One animal only, *T. taeniatus* 164, shows an asymmetric brain. It contains only one telencephalon hemisphere. In the discussion of the relative positions of the two embryonic rudiments, we saw that it was very probable that in a number of cases the graft certainly did not consist of the *medial* part of the dorsal blastoporal lip. Hence, it is evident that *in several cases a mediolateral regulation from an asymmetric into a bilaterally symmetric archenteron roof has taken place.* This mediolateral regulation has been observed already by several authors.

II. *Cranio-caudal regulation*†

Before we can analyse this regulation we must first discuss the development of the archenteron roof (notochord and myotomes) and neural differentiations (neural tube, dorsal and tail fin and ganglia) in the trunk and tail. I shall give a short survey.

(*a*) A first group contains a '*complete*'‡ secondary archenteron roof, corresponding either with a complete nervous system (neural tube, dorsal and tail fin, etc.) or with a nervous system which has fused with the primary one in the caudal part of trunk or tail. The first situation is present in five animals, the second in two. Further, I observed four animals in which the secondary notochord fuses with the primary, but in which we find a complete neural tube and dorsal and tail fin. In all these cases there has probably been, originally, a 'complete' archenteron roof which has, however, secondarily fused with the primary in the four animals last mentioned.

(*b*) In a second group the notochord and the neural tube as well fuse at a certain level with the corresponding organs of the primary rudiment. In a number of cases the fusion of the neural tubes is situated anterior to the fusion of the notochords. In those cases the neural tube shows, however, a distinct double structure up to the point of fusion of the two notochords. In other animals the points of fusion lie at the same level. In these animals it cannot be decided whether the secondary archenteron roof was originally complete or not. In four cases, however, there are some indications that it was: the region of fusion does not lie in front of the tail-root, and the secondary tail fin extends to the tip of the tail. Another animal represents a transition to the situation in the remaining cases of this group. In this animal the secondary notochord fuses with the primary in the tail, whereas the neural tube and fin are already united in the caudal trunk region. Finally, the two remaining animals show a fusion of primary and secondary notochord, neural tube and fin in the caudal trunk region. Probably the first four or five animals of this group represent cases in which the original 'complete' archenteron roof and neural tube have fused only secondarily with the primary ones as in the former group.

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* We must here leave out of consideration the incomplete development of the sense organs (cf. p. 170), and restrict ourselves to the development of the brain itself.

† By *a cranio-caudal regulation* is meant *a shifting in the regional structure of the organizer along the longitudinal axis* in consequence of an internal regulation of its components (or an assimilatory addition of missing parts from the environment).

‡ 'Complete' signifies here that the archenteron roof or neural tube shows no interruption from its front end (a certain level in head or trunk) to the end of the tail.

(*c*) A third group consists of animals in which the notochord ends blindly in trunk or tail. Here certainly one has to do with an incomplete archenteron roof. The behaviour of the secondary neural tube is variable. In some cases it fuses with the primary; in two animals in front of the end of the notochord, in one, however, much more caudad. Caudally to the end of the notochord the neural tube has been reduced to a considerably narrowed, even rudimentary, cell column. The secondary dorsal fin extends, however, to the tip of the tail. In the remaining nine animals the neural tube extends farther caudad than the notochord, in one case even to the tip of the tail. The secondary fin has been completely developed in almost all these animals.

In this series it is, indeed, unlikely that the 'complete' dorsal blastoporal lip (up to the border of the animal field) has been grafted in the large number of cases, 50 % and more, in which a 'complete' archenteron roof has been formed. This probably points to *an internal cranio-caudal regulation** of the graft. Probably this regulation has occurred mainly in those cases in which the transplantation has been performed at the *early* gastrula stage. Moreover, the almost complete absence of local defects in the axial organs (notochord, myotomes and spinal cord) argues in favour of the presence of a mediolateral and *a cranio-caudal* regulation of local defects in the original archenteron roof, defects which were surely present in several cases.

The relative cranio-caudal position of the two rudiments varies only within certain limits. In a number of cases the trunk organs of the two rudiments are situated almost at the same level, whereas in other animals the differences are more important. The distance between the end of the pronephroi of primary and secondary rudiments, however, never exceeds a value of one-fifth of the length of the trunk. This is remarkably little when one remembers that a very small error in the localization of the graft at the early gastrula stage causes a very markedly altered position of the graft in the larva. The original position of the graft has probably shown much greater variation with respect to the corresponding levels of the host than the values here observed. This points to *a cranio-caudal regulative effect of the environment* (ventral ectoderm, regional influences of the primary rudiment, etc.), by which more corresponding structures are formed in primary and secondary rudiment at a same level of the trunk.

Finally, a last aspect of the cranio-caudal regulation has to be discussed. Some animals in which I could also determine the distances between corresponding points situated farther forward in the primary and secondary rudiments (ear vesicles, tip of the notochord), show that these distances are markedly greater than those between the ends of the pronephroi. This means that the secondary rudiment is shorter than the primary one. As the distances between corresponding points decrease in a caudal direction, and the tips of the tails coincide, we have to do with a shortened embryo, every part of which has been proportionally decreased in length. These secondary rudiments show, however, an entirely harmonious form, the lengths and diameters of every part of the brain being in correct proportion (cf. Figs. 10–15). *A cranio-caudal regulation (shortening) must, therefore, have gone hand in hand with a mediolateral regulation, and vice versa.*

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* Here, a cranio-caudal regulation means a regulation by which the whole, or part, of the cell material of the archenteron roof missing caudally has been added to the graft by regulation so that again a new whole has been formed as completely as possible.

However, these considerations concerning the cranio-caudal regulation still have a speculative character, and must be investigated further.

The last animals of group (*c*) demonstrate a very interesting fact. Probably during gastrulation the archenteron roof was not complete; its caudal part failed here. It invaginated and shifted beneath the ectoderm up to its definitive position; therefore it has been only temporarily in contact with the caudal ectoderm. This sufficed, however, to induce a dorsal or tail fin and in many cases even a rudimentary neural tube.* The *tail fin*, normally developing from the neural folds, signifies a *lateral* neural differentiation, whereas the *neural tube* corresponds to a more *medial* differentiation of the neural induction. A very short inductive action may, therefore, evoke the formation of a lateral differentiation only, whereas with a longer duration of the induction more medial differentiations may appear as well. This view agrees very well with that of Raven & Kloos (1945), who assume a quantitative difference determining the mediolateral differentiation of the neural induction. Moreover, these animals show that the induction by the archenteron roof begins as soon as it has come into contact with the ectoderm.

(*b*) *Correlations between the regional differentiations in the archenteron roof and induced nervous system*

It is a striking fact that the boundary between prechordal and chordo-mesodermal plate coincides with the boundary between arch- and deuterencephalon in all experimental animals. The question arises: Is the regional differentiation of the archenteron roof a primary or a secondary one, i.e. do the presumptive areas of the archenteron roof induce always the same brain parts or can the differentiation of the archenteron roof be influenced secondarily by the differentiation of the induced neural plate? We know from Holtfreter's exogastrulation experiments (1933*d*) that the archenteron roof can differentiate to a high degree without any influence of the overlying ectoderm. Therefore we must conclude that in the archenteron roof the most important boundaries between its separate organ fields (notochord, prechordal plate, myotomes, etc.) can be determined independently. A definite part of the archenteron roof does not, however, always induce the same neural structure, depending on the position of the graft in the host (Spemann, 1927*a*, *b*, 1931*a*;

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* The possibility must, however, be taken into account that in these cases the caudal part of the graft has not differentiated into notochord and myotomes, notwithstanding the fact that it has had inductive power.

Bautzmann, 1928*a*, *b*, 1929; Chuang, 1939, 1940). The above-mentioned considerations on the cranio-caudal regulation have made probable that this holds true also in my experiments. We have to conclude, therefore, that the differentiation of the graft and of the induced neural plate change in a corresponding manner, so that definite boundaries in the archenteron roof and neural plate finally coincide in all cases. This phenomenon may be explained in two ways: (1) by a primary segregation of the presumptive areas in the archenteron roof by influences from the environment (primary rudiment, etc.), each part then inducing the corresponding part of the brain, (2) by a secondary influence of the regional structure of the induced neural plate on the final differentiation of the archenteron roof. This last possibility definitely cannot be excluded.

These considerations also hold good for the formation of the cartilages; their form always shows close correlation with the form of the brain induced. Moreover, the differentiation of the neurocranium occurs very late—several embryos described above did not yet show a sufficient cartilage differentiation (cf. Figs. 12, 13*a*). This points strongly to a secondary determination of the skeleton by the nervous system itself. Hence the neurocranium does not provide any indication of the original structure of the graft.

(*c*) *'Primary' and 'secondary' parts of the brain*

The experimental animals described above demonstrate that in spite of strong abnormalities in certain brain parts, a basal structure of these brain parts is always present. Certain structures often show defects or an incomplete development, whereas others always differentiate, when that part of the brain in question is formed. We may, therefore, distinguish between 'primary' structures, which are always present, and 'secondary' structures, which are more variable. I will give some examples.

The important nuclei with their commissures, for instance, may be considered as 'primary' structures. For example, in the telencephalon, the primordia hippocampi (dorsally) with the commissura pallii anterior and the basal regions (ventrally) with the commissura anterior. In the diencephalon, the corpora habenulae (dorsally) with the commissura habenularis, the primary optic centres in the side walls and the commissura postoptica (ventrally). (The epiphysis and the tela chorioidea are also rather constant structures.) In the mesencephalon, the tectum opticum with the commissura posterior, the tegmentum and the commissura tuberculum posterior. In the metencephalon, the lateral auricles with the commissura cerebellaris and the ventral nuclei with the commissura ventralis. Finally, in the myelencephalon, the primary nuclei of the cephalic nerves V–X, Mauthner's cells, the tela chorioidea and the ventral commissures.

Parts to be considered as more variable are: In the telencephalon the connective nerves* with the nasal pits (dependent on the presence of these latter), the nasal pit* itself (secondarily formed after direct contact of the telencephalon with the cephalic ectoderm), and the paired structure of this part of the brain (being lost when the telencephalon is reduced) (cf. Figs. 11*b*, 12). In the diencephalon, the eyes and the chiasma opticum. In spite of the entire absence of one or both eyes, the diencephalon shows a normal basal structure (cf. Figs. 12, 13*a*). Then the infundibulum: parallel with a ventral displacement of the eyes this part shows all intermediate stages between a slight reduction up to a complete absence (cf. Figs. 12*a*, 11*a*, *b*). Finally the hypophysis: this may be lacking even with a completely differentiated infundibulum (cf. Fig. 12*a*). In the myelencephalon, the ear vesicles: these are, indeed, very constant, but their size is dependent on the number of neuromeres of the myelencephalon present (cf. pp. 172, 173). A reduced ear vesicle develops even when only the most caudal neuromeres (from the ninth or eighth nerve on) are present (cf. Figs. 14*d*, 15).

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* They represent rather constant formations in the case where a telencephalon is present.

It is of great importance to consider the 'ground plan' of the 'primary' parts of the brain segments. Thus we get a clearer and simpler picture than in the presence of all the 'secondary' parts (cf. Table 1).

Table 1.

Part of brain	Dorsolateral centre	Commissure	Ventrolateral centre	Commissure
Telencephalon	Prim. hippocampi	Com. pallii anterior	Basal regions	Com. anterior
Diencephalon	Corpora habenulae	Com. habenularis	Thalamus opticus	Com. postoptica
Mesencephalon	Tectum opticum	Com. posterior	Tegmentum	Com. tuberculum posterior
Metencephalon	Cerebellum, auricle (dorsal part)	Com. cerebellaris	Auricle (ventral part)	Com. ventralis
Myelencephalon	Dorsal centres of cephalic nerves V–X	—	Ventral centres of cephalic nerves V–X	Band of ventral commissures
Spinal cord	Segmental dorsal centres	—	Segmental ventral centres	Segmental ventral commissures

The first four parts of the brain show a similar ground plan, viz. dorsal centres with dorsal commissures and ventral centres with ventral commissures. The dorsal commissures are much smaller than the ventral. In the absence of the recessus opticus (and eyes) and of the infundibulum, these latter form one continual band from the tip of the brain back to the spinal cord and farther. The myelencephalon and spinal cord segments differ from the first four parts of the brain by the absence of the dorsal commissures. With the exception of the epithelial roof of the fourth ventricle in the myelencephalon, these parts resemble each other in many respects.

The following structures are formed by the successive parts of the brain in addition to this ground plan. Telencephalon: a paired structure with two hemispheres, two lateral ventricles, etc., and connective nerves with the nasal pits. Diencephalon: the paired eyes with nervi optici, chiasma opticum and recessus opticus, the ventral infundibulum and hypophysis, and the dorsal paraphysis, tela chorioidea and epiphysis. Myelencephalon: the tela chorioidea of the roof of the fourth ventricle.

The causal analysis of the development of the nervous system may probably be divided into two fields of investigation: (1) *the determination of the 'ground plan' of the central nervous system and* (2) *the more dependent development of the specific formations of the separate parts of the brain.*

(*d*) *Causal relations between various parts of the archenteron roof, ectoderm and neural plate*

Extensive investigations have already been made on the last subject, e.g. the development and determination of the paired eyes, the development of the epiphysis, etc.

These experiments again demonstrate clearly that the development of the nasal pit is dependent on the presence of the telencephalon (paired or unpaired) (cf. Figs. 11*b*, 12*a*, 13*a*).

The rudiment of the infundibulum is originally situated far forward, about on a level with the anterior border of the foregut and the presumptive rudiment of the ectodermal stomodaeal invagination. Fig. 11*a* and *b* show that a *ventral* displacement of the eyes (ventral position of the eyes, synophthalmia or cyclopia) is associated with the *absence* of the infundibulum. This fact permits of two interpretations: (1) The ventral position of the eyes and the absence of the infundibulum have been caused by one and the same local defect in the neural plate and primarily in the archenteron roof. (2) The ventral displacement of the eye rudiments has prevented a direct contact between the neural plate, the archenteron roof and the cephalic ectoderm, which is necessary to the final determination of the infundibulum. In that case, the formation of the primary eye vesicles must precede the final determination of the infundibulum. The *presence* of an infundibulum in the *absence* of one or both eyes and of the chiasma opticum (Fig. 12*a*) argues in favour of the second interpretation. Moreover, the lack of an infundibulum in the absence of an entodermal foregut (Fig. 13*a*) also points to the necessity of a direct contact between the neural plate and the entodermal foregut. Finally, the *absence* of infundibulum and hypophysis in the primary embryos (90 and 91/4), in which, after the normal formation of the neural plate, the middle part of the brain has *secondarily* been everted instead of inverted, forms a strong argument in favour of a late determination of the infundibulum by a direct contact between archenteron roof and neural plate. Fig. 12*a* finally demonstrates that the development of the infundibulum is independent of the presence of the hypophysis.

I never found a hypophysis in the absence of an infundibulum. The hypophysis normally develops from ectoderm situated on the borderline of brain and foregut. In all the embryos in which a hypophysis fails, either a large quantity of mesenchyme, or one or two ventral eyes fill up the large space between infundibulum, foregut and cephalic ectoderm (cf. Figs. 11*b*, 12*a*). A direct contact between brain and ectoderm does not suffice in the formation of the hypophysis (Fig. 13*a*). Therefore, I suppose that a triple contact between brain, foregut and ectoderm is required for the determination of the hypophysis.

The formation of the chiasma is, self-evidently, dependent on the presence of *paired* eyes. A real chiasma fails in case of marked synophthalmia or cyclopia (cf. Figs. 11*a*, *b*).

The formation of the ear vesicles is related to the presence of the caudal segments of the myelencephalon. I cannot decide whether they are induced, either by this part of the neural plate, or by the corresponding neural folds. The formation of the unpaired, bilaterally symmetrical ear vesicles in Figs. 14*d* and 15 can be explained in two ways: (1) by assuming an inductive action on the adjacent ectoderm by the caudal neuromeres of the myelencephalon—from the eighth or ninth nerve on—situated mediocranially in these rudimentary nervous systems; (2) by supposing an inductive action by the corresponding parts of the neural folds, now fused in front of the neural plate. Fig. 15 finally shows that even the most caudal part of the myelencephalon, from the ninth and tenth nerve on, can induce a rudimentary ear vesicle.

In Fig. 13*a*, in which the entodermal foregut fails, the ectodermal stomodaeal invagination is also absent. This agrees with the observations of Stroër (1933), Woerdeman (1943) and Nieuwkoop (1946). In my experimental animals there exists a striking parallelism between the presence of a telencephalon (complete or reduced) and the formation of an ectodermal stomodaeal invagination. A still very speculative explanation may be that the foremost part of the brain and the ectodermal stomodaeum are determined by the same portion of the organizer, viz. the presumptive prechordal mesentoderm.

(*e*) *Correlations between sense organs and brain and between the various parts of the brain*

These incomplete nervous systems give a further point of attack for the analysis of the correlations between the sense organs and the brain and between the different parts of the brain. For a thorough analysis it is, however, necessary that the histological structure of the brain (nuclei and tracts) can be clearly identified. Sad to relate this material does not sufficiently meet this requirement, as only a general staining method has been used here. Therefore, I can only record some generalities.

It is evident that the primary and secondary centres of the sense organs are formed and differentiated in a normal way in the absence of the function of the corresponding sense organs, and even in the absence of the whole organ. When the nasal pit is rudimentary, without communication with the outer world or the mouth cavity (cf. Figs. 11*b*, 12*a*) or even when the nasal pits are absent (cf. Fig. 13*a*), the roof of the diencephalon contains normal corpora habenulae with their commissure. In the absence of the eyes there is a normal thalamus opticus in the diencephalon and tectum opticum in the mesencephalon (cf. Fig. 13*a*, *b*).

The commissura postoptica has hardly a smaller diameter in the absence of the eyes and chiasma than in the case of normal functioning eyes. This proves that the commissura postoptica contains none or only a few primary optic fibres in the Urodeles.

After specific staining these incomplete nervous systems may form a very important source of data for the causal analysis of the development of the tracts and nuclei in the nervous system, especially in conjunction with the exhaustive investigation of the normal development by Herrick (1937–9). It is my intention to submit the development of incomplete embryonic rudiments to a detailed investigation in this direction also.

(*f*) *New data on the regional determination of the central nervous system*

All the experimental animals show the important fact that the induced parts of the brain have normal relative proportions. In many cases they are, moreover, hardly smaller than the corresponding parts of the primary rudiment[*] (cf. Table 2).

Table 2. *Areas of the successive parts of the brain and sense organs in sagittal projection with an enlargement of* 125 × *expressed in* cm^2. (*in absolute value and in percentage of the corresponding values of the primary rudiment*).

No. of animal	Area of telen-cephalon		Area of nasal pit		Area of dien-cephalon		Area of eye		Area of mesen-cephalon		Area of rhomben-cephalon		Area of ear vesicle		Area of brain	
	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%
97/9	66	71	30	103	96	103	163	97	88	111	190	90	133	134	440	93
149/8	70*	70	27*	75	72*	73	127*	64	76*	75	190*	75	110	90	408*	74
158/9	61*	66	16*	57	73*	56	90*	62	74	97	222	104	114	97	430*	84
164	102	(167)	56	108	203	(271)	188	104	96	101	196	73	227	(198)	597	(119)
I 164	61†		52		75†		182		95		269		115		500	
113/5	33	48	31	135	37‡	41	95	83	70	113	189	90	178	136	329	(76)
92/4	42	46	22	73	80‡	73	166	86	104	121	227	100	144	135	453	(88)
156/5	12	34	10	83	71	86	60	67	22	51	138	71	79	111	243	(68)
148/7	28	80	7	37	69	100	57	67	50	98	163	124	79	102	310	(108)
I 145/6	8		7		99		—		73½		240		99		420	
91/4	—		—		34‡	§	27	§	36	§	160	§	105	§	230	§
154/5	—		—		20‡	20	—		51	56	154	68	61	76	225	(46½)
109/10	—		—		74	106	—		30	59	161	99	102	123	265	(80)
90	—		—		—		—		63§		192	140	125	116	225	(78)
102/4	—		—		—		—		39	25	204	104	102	84	243	(57)
100/1	—		—		—		—		—		284	141	144	101	284	(59)
96/101	—		—		—		—		—		154	62	92	75	154	(30)
150/48	—		—		—		—		—		207	(74)	116	125	207	(38)
114/5	—		—		—		—		—		159	(59)	121	97	159	(29)
98/9	—		—		—		—		—		133	(64)	83	89	133	(30)
106/7	—		—		—		—		—		157	(52)	98	67	157	(25)
105/7	—		—		—		—		—		86	(34)	188	91	86	(16)
93/4	—		—		—		—		—		117	(66)	109	98	117	(34)
163/2	—		—		—		—		—		127	(45)	195	116	127	(19)
161/2	—		—		—		—		—		60	(23)	98	74	60	(10)
111/2	—		—		—		—		—		111	(45)	61	45	111	(22)
103/4	—		—		—		—		—		81	(31)	7	5	81	(14)

* Too low values caused by deformation of the reconstruction.

† Wrong values caused by reduced size of corresponding parts of brain of primary rudiment.

‡ Too low values caused by the absence of an infundibulum.

§ Values which could not be calculated owing to strong abnormalities in the primary rudiment.

Percentages given in parentheses denote values not directly comparable (primary rudiment with complete brain or parts of the brain, secondary rudiment with only a portion of the corresponding part(s).

The discussion of the cranio-caudal regulation suggests that the presumptive archenteron roof (dorsal blastoporal lip) tends to regulate (internally or by assimilation) the caudal missing parts in order to form a new whole. This whole induces a nervous system which is always 'complete'[†], in so far as only the foremost parts may be lacking. This means that *the nervous system is completely formed up to a certain*

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[*] Primary and secondary rudiment always show the same degree of differentiation. This relation does not hold between different duplicitas. Although all the animals are of the same age (reared during a same number of days), the individual development and differentiation are somewhat variable (cf. Figs. 10*b*, 12*b*).

[†] Cf. n. ‡ on p. 167.

level. This level may be situated either exactly between two successive parts of the brain or within one of the parts. In the first case all the parts of the brain formed are entirely normal (at least as far as the 'primary' structures are concerned, the 'secondary' structures may be partly or wholly lacking). In the second case all the parts of the brain with the exception of the most anterior part are 'complete'; the latter, however, tends to form a reduced image of the normal.* The most anterior part of the brain can be formed in any degree of reduced size without any influence upon the following parts.

From this material I can frame a complete regression series of the nervous system: normal paired telencephalon—indication of a paired structure of the telencephalon—unpaired telencephalon, reduced in size down to a very small rudiment—telencephalon lacking, diencephalon complete, etc., etc.

This characteristic development of the nervous systems of the duplicitas allows the following conclusions: (1) *The central nervous system is determined in a large number of successive zones*, at least seven in the brain alone, viz. tel-, di-, mes-, met- and three zones in the myelencephalon. (2) *All these brain parts are qualitatively 'equivalent'*; each of them can form the most anterior part of the brain induced. This implies that the formation of any part is independent of the presence of another part anterior to it. The view that the mesencephalon is formed by an overlapping of the arch- and the deuterencephalic determination zones only cannot be upheld, since the mesencephalon can form the most anterior part of the brain. (3) *Each of these parts of the brain must be determined by a* (qualitatively or quantitatively) *different value of the organizing 'agent'.*

The presence of this large number of successive zones of determination can with difficulty be reconciled with the view of Lehmann (1942). He supposes that the prechordal plate is responsible for the determination of the arch-encephalic and the chordo-mesodermal plate for the deutero-encephalic part of the brain. This subdivision of the archenteron roof seems to me insufficient to explain the presence of all these successive 'equivalent' zones. A much finer division in zones of determination seems to be required.†

Finally, I will point out the interesting parallelism between the position of the boundaries of the successive zones of determination and the anatomical, regional structure of the brain. The brain as well as the spinal cord is supposed to consist of a number of neuromeres. Tel- and metencephalon correspond to one neuromere, di- and mesencephalon consist of two and finally the myelencephalon is thought to be formed from a number of neuromeres. This supposition agrees in the main with the configuration of the zones of determination in the brain.

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* It is possible that it forms either a reduced image of the whole part of the brain, or its caudal part (segment) only in the case of this part of the brain consists of more segments.

† I will illustrate the determination of an incomplete nervous system by a domestic analogy. Certain portions are successively weighed out from the neural tissue present, beginning with the most caudal part and continuing in a caudo-cranial order till the remaining tissue is no longer sufficient to form the next part. Then this remaining tissue forms a reduced image of this part. Parts still further forward simply get no material at all.

GENERAL CONSIDERATIONS OF THE REGIONAL DETERMINATION OF THE CENTRAL NERVOUS SYSTEM: A NEW WORKING HYPOTHESIS TO FURTHER ANALYSIS OF THIS DETERMINATION PROCESS

The central nervous system consists of a large number of clearly discernible parts which have a definite sequence. Morphologically, the various parts of the brain differ markedly from each other. With respect to the 'primary' structures the preceding chapter has shown that, in fact, the parts of the brain resemble one another in many respects. The 'secondary' structures, however, determine to a marked extent the external form of the brain.

From the literature we know that the *presumptive* neural plate of the early gastrula shows no tendency to differentiate as neural tissue (Holtfreter, 1933). Moreover, pronounced differences in reactive power to neural induction between distinct parts of the animal field are probably still absent (cf. p. 177). The neural differentiation tendencies appear immediately after the formation of the substrate; they are evoked by the archenteron roof.

Considering the temporal course of the determination process, we now know that the determination begins as soon as the archenteron roof makes contact with the ectoderm. This contact temporally and spatially extends from behind forwards. The ectoderm is reactive to influences of the archenteron roof only during a short period (certain degree of maturity) (Mangold, 1928, 1929), whereas the presumptive archenteron roof itself shows inductive power from the uncleaved egg (Mayer, 1939) till the early larva stage (Holtfreter, 1933). The reactivity of the ectoderm quickly decreases—also in a caudo-cranial direction—with increasing age of the reaction system (Machemer, 1930, 1932; Lehmann, 1932; Holtfreter, 1936, etc.). These facts together prove that the period during which the induction can really act is almost the same in all parts of the presumptive neural plate.

The power of internal regulation of the presumptive archenteron roof, markedly present at the early gastrula stage, soon decreases with the progress of development (Mangold & Spemann, 1927; Mayer, 1935; Lehmann, 1938, etc.). The power of regulation in the ectoderm lasts longer (Daltrop, 1933), but it also decreases quickly during neurulation. Parallel with this decrease of the power of regulation, more and more pronounced differentiation tendencies appear. The local differences in the inductor (archenteron roof) become more pronounced, probably also its inductive power increases, so that the regional influence of the reaction system (ectoderm) seems to be more and more repressed (cf. Spemann (1931) and Marx (1925) with Bautzmann (1928, 1933), O. Mangold (1929, 1933), Holtfreter (1933) and Töndury (1936)). In my opinion, the character of the induction process changes gradually during gastrulation, viz. from a 'regulative' into a 'mosaic' induction (cf. p. 181). This implies that the successive stages of the determination process must be analysed separately—a very important fact which, however, has received little consideration hitherto.

As we have seen above, the ectoderm has no tendency towards neural differentiation before the formation of the substrate, whereas the archenteron roof itself at an early stage shows strong differentiation tendencies, to some extent localized. The exogastrulation experiments of Holtfreter (1933) have shown that, although the archenteron roof can develop its spatial pattern to a large extent without any influence of the ectoderm, its final differentiation is determined also by the overlying ectoderm. There exists a mutual interaction between archenteron roof and neural plate.

Now we come to the question whether the spatial pattern of the neural plate is determined by the regional structure of the archenteron roof only, or by the regional structures of archenteron roof and presumptive ectoderm together. The opinions are very conflicting concerning the presence of a regional structure in the presumptive ectoderm, whereas they entirely agree about a regional structure in the archenteron roof. In my opinion the arguments of Holtfreter (1933–6)—e.g. the non-appearance of an original regional structure of the ectoderm in explants, in the ectodermal grafts placed on the ento-mesoderm of the exogastrula and in those transplanted into the flank of older larvae—against a regional structure of the ectoderm are not entirely conclusive. He has called no attention to the possibility of a disappearance of a weak regional structure of the ectoderm in explants in consequence of an internal regulation or a harmful influence of the milieu, nor to the fact that the regional influences of the archenteron roof at older developmental stages are probably much more pronounced, and different in nature from those at early stages, so that a weak regional structure of the ectoderm is masked. In the literature there are, moreover, several arguments in favour of a weak regional structure of the ectoderm. A first argument is the formation of an intermediate state between brain and spinal cord by a trunk inductor (in the place of the normal head inductor) in cephalic ectoderm (Hall, 1932, 1937). The formation of bilaterally symmetrical, well-differentiated, regionally structured, forebrain inductions by a heterogeneous inductor (liver) in explanted presumptive ectoderm (Chuang, 1938) can with difficulty be understood without assuming a regional structure in the latter. Finally, the gradual decline of the mesodermal differentiation potencies from the dorsal blastoporal lip in ventral (vegetative) and animal direction (even into the presumptive ectoderm), and of the ectodermal differentiation potencies from the animal pole even into the vegetative field probably agree respectively with a decline in the intensity of the inductive action of the archenteron roof, as well as with the presence of a decline in reactive power to neural induction in the ectoderm.

In the duplicitas embryos we must also take into account the existence of regional influences of the environment upon the determination of the secondary rudiment, so that here an interaction between the strong regional structure of the graft, the weak regional structure of the presumptive ectoderm, and the regional influences of the primary embryonic rudiment is present.

Now the question arises whether the regional structures of the archenteron roof and presumptive ectoderm are based on mosaics of qualitatively different areas, or on quantitative gradients. Here opinions differ strongly, e.g. Toivonen (1938), Chuang (1938, 1939) and Lehmann (1938–42) accept the first idea in the main, whereas Dalcq (1940, 1941) and Pasteels (1938–41) defend a theory based on purely quantitative influences.

The 'qualitative hypothesis' is based on the phase-specific action of Li (Lehmann, 1938), on the specificity of heterogeneous inductors, the activities of which are identified with inductive substances from corresponding parts of the archenteron roof (Toivonen, 1940; Lehmann, 1942), etc. This hypothesis is also supported by experiments of Shen (1939, 1942), who found only quantitative differences in the nervous tissue induced by increasing concentration of a water-soluble hydrocarbon. Waddington, Needham & Brachet (1936), Weiss (1935) and Woerdeman (1937), however, consider the actions of the abnormal inductors as a non-specific activation of the reaction system only.

A large number of phenomena has been observed within the last ten years—e.g. the variability in the structure of the heterogeneous inductions (Toivonen, 1938; Chuang, 1938–9), the qualitative differences in the inductions with a normal and a killed inductor (Holtfreter, 1932–3), the general reduction of the morphogenetic potential of the archenteron roof after Li treatment (Pasteels, 1945), the general positive effect of NaSCN (Ranzi & Tamini, 1940), the general stimulating effect with a weak, and the depressing effect with a stronger irradiation with ultra-violet light (Brandes, 1940), the appearance of a series of abnormalities extending in cranio-caudal and dorsoventral directions after centrifuging eggs of *Rana fusca* and *Discoglossus* (Pasteels, 1940), similar effects of supranormal temperatures and over-ripening of the eggs (Hoadley, 1938), the internal regulation in the grafted archenteron roof, etc. Very complicated theories are necessary to explain all these phenomena on the basis of a qualitative character of the induction process. On the other hand, a simple decrease or increase of the quantity of the inductive 'agent' may explain all these facts if the nature of the induction process is purely quantitative (at least during the beginning of the determination process).

Different parts of the archenteron roof can evoke the formation of different neural structures in the same ectoderm. This suggests strongly that the original structure of the ectoderm also is purely quantitative in character.

Summarizing, we have probably to do with two gradient fields, a mesodermal, originally extending from the dorsal side in ventral and animal directions, and an ectodermal, extending from the animal pole in vegetative direction (cf. Fig. 16*a*). After invagination of the marginal zone both gradients decline in the cranio-caudal direction (cf. Fig. 16*b*).

In an equilibrium reaction between the 'substances' underlying these gradients, a gradient of the 'reaction product' is formed, which shows a similar cranio-caudal direction of decline (more or less steep than the mesodermal gradient) (cf. Fig. 16*c*). Each part of the brain now corresponds to a distinct level of the gradient of the reaction product.

However, in contradistinction to the situation in cranio-caudal direction, each *transverse* zone is characterized by a *constant* value of the ectodermal and a *steep decline* of the mesodermal gradient.* In an equilibrium reaction between both gradients, the gradient of the 'reaction product' will, therefore, show a less steep decline with respect to the mesodermal gradient *in the mediolateral direction* than *in the cranio-caudal direction*. The presumptive parts of the brain will, therefore, also show a V-shaped form, similarly directed to those of the archenteron roof, but with a greater angle between the two lines of the V (cf. Fig. 17*c*, *d*). The investigations of Manchot (1929) and Daltrop (1933) have shown that the presumptive areas of the various parts of the brain actually are broadly V-shaped in the early neural plate.

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* The mesodermal gradient, decreasing equally in almost all directions in the marginal zone of the early gastrula, is very markedly stretched in a longitudinal direction after invagination. In the invaginated archenteron roof we may, therefore, expect steep V-shaped zones of equal value of the mesodermal gradient, the tips of which point caudally (cf. Fig. 17*a*, *b*).

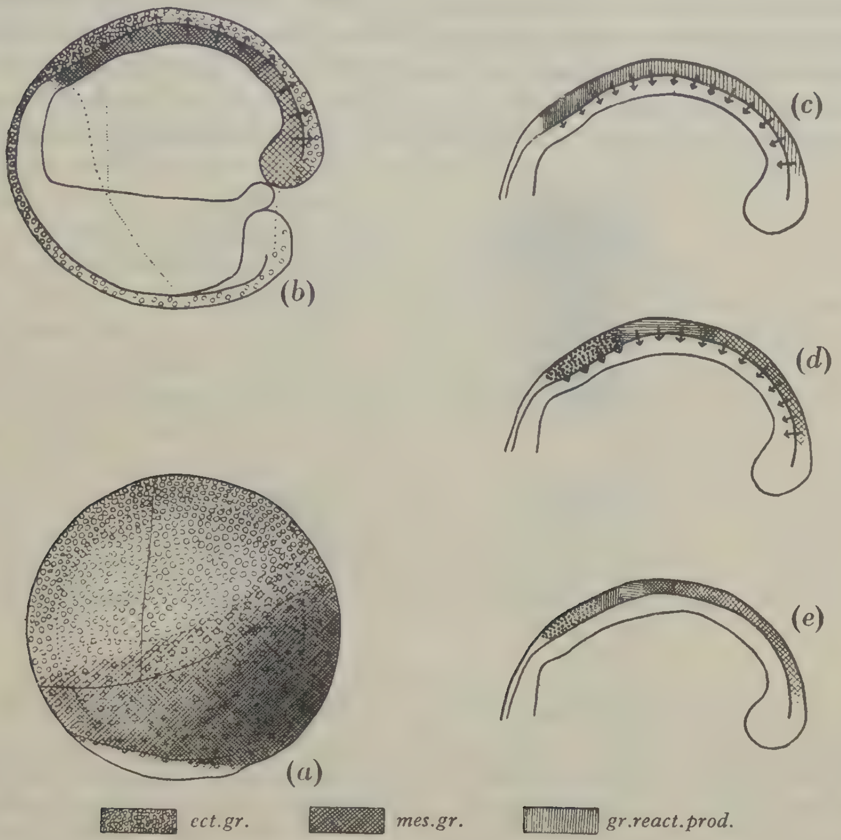

Fig. 16. (*a*) Diagram of distribution of ectodermal and mesodermal differentiation potencies at early gastrula stage; external view. Continual lines: boundaries between areas of vegetative field, marginal zone and presumptive ectoderm and neural plate. Dotted lines: projection of blastocoel. (*b*) Distribution of both differentiation potencies at slit-blastopore stage; medial section. Dotted lines: projection of boundaries of invaginated marginal zone (Vogt, 1929). Arrows: direction of diffusion of inductive agent. (*c*) Distribution of 'reaction product' of ecto- and mesodermal gradients in presumptive neural plate; medial section through archenteron roof and neural plate. Small arrows: reciprocal influence of 'reaction product' on underlying archenteron roof. (*d*) and (*e*) Gradual subdivision of neural plate into separate organ fields; distribution of various 'reaction products'. *Ect.gr.* ectodermal gradient; *mes.gr.* mesodermal gradient; *gr.react.prod.* gradient of 'reaction product'.

Bautzmann (1929) had already pointed out the difficulty of reconciling qualitatively different formations with quantitative differences in the inductor.

Lehmann (1938*b*) stated that the regional pattern of the archenteron roof, hardly determined at the early gastrula stage, must be definitely fixed during gastrulation, since pronounced regional differences are present in the archenteron roof of the early neurula. In my opinion, the same holds good for the ectoderm during the determination to neural plate. These regional differences must be based ultimately on qualitatively different chemical structures.

How can we imagine the transition of quantitative into qualitative differences? This is still entirely speculative. The very first beginning of the origin of qualitative differences may be the formation of closely related chemical substances,* which, however, may act in markedly different ways. Each determination process is probably composed of a long chain of chemical reactions. The moment of the actual appearance of qualitative differences may, therefore, vary a great deal; it may occur either at the beginning (qualitative, 'mosaic' induction), or at an intermediate stage, or, finally, at the end (quantitative, 'regulative' induction) of the determination process.

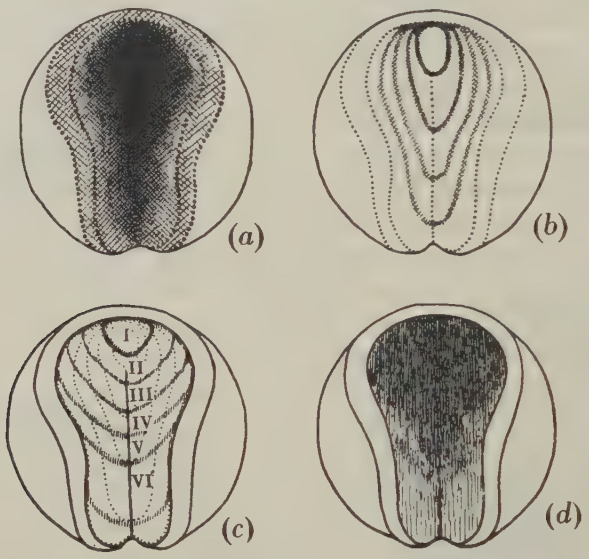

Fig. 17 (*a*) and (*b*). Diagrams of spatial distribution of mesodermal gradient in archenteron roof; dorsal view. External form of neural plate: dotted. (*b*) Lines of equal concentration. (*c*) and (*d*). Diagrams of spatial distribution of gradient of 'reaction product'. (*c*) Lines of equal concentration; similar lines of the mesodermal gradient: dotted. Roman numerals: successive parts of brain and spinal cord.

Here lies, in my opinion, the possibility of reconciling the contradictory views of a 'quantitative' or a 'qualitative' hypothesis in the induction of the nervous system. Similarly, the special character of the prechordal plate may be explained by assuming a *very early* appearance of qualitative differences in this part of the archenteron roof.

This train of thoughts also meets with the serious objection which can be raised against the view that a sharp order of threshold values of the gradient is the basis of the regional structure of the archenteron roof and neural plate. It is a well-known fact that every induction field has the character of a 'Zerstreuungskreis', and that the various fields partly overlap each other. Two gradient fields reacting with each other, will surely not show a sharp mosaic of areas with qualitatively different chemical structures. Only with optimal proportions will one of the 'reaction products' be formed in excess, whereas in intermediate areas mixtures of various 'reaction products' will be formed. During the determination process the original determination field is gradually divided into an increasing number of separate induction fields, which, originally, show very vague boundaries, but which are later delimited more and more sharply (cf. Fig. 16*d*, *e*).

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* Different proportions of two components reacting with each other may cause the appearance of closely related, but chemically different reaction products.

We may conclude that the character of the induction process probably changes fundamentally from a purely quantitative into a more and more qualitative one during the course of the determination process (cf. p. 176).

The determination of an embryonic rudiment by a grafted dorsal blastoporal lip probably begins with a redistribution of the mesodermal gradient (mediolateral and cranio-caudal regulation), the intensity of which is dependent on the age of the graft. Then, the new mesodermal gradient reacts with the gradient of the overlying ectoderm, by which in the course of several reactions different chemical structures are formed locally. These different 'reaction products', increasing in number, gradually determine the final spatial structure of the embryonic rudiment, primarily determining the structure of the neural plate, but possibly secondarily also influencing the differentiation of the archenteron roof.

With this working hypothesis I hope to be able to augment our knowledge concerning the real nature of the determination process of the regional structure of the central nervous system.

SUMMARY

1. With the aseptic operation method of Woerdeman (1930) it is possible to rear duplicitas larvae, formed after grafting of the dorsal blastoporal lip in the place of the ventral one at the early gastrula stage (Spemann, 1918), till an age of about 4 weeks, a time at which they have completely consumed their yolk material.

2. Various forces acting in the normal development and in the development of the duplicitas larvae are discussed in connexion with the external form and the internal organization of the latter.

3. The sources of errors in the method of graphic reconstruction of the brain used in this investigation are checked and their elimination is discussed.

4. The structure of the duplicitas shows that there exists a mediolateral and probably also a cranio-caudal regulation in grafts of the dorsal blastoporal lip of the early gastrula.

5. In the brain one may distinguish between 'primary' structures which are always present, and 'secondary' structures which are more variable. Therefore, one may probably divide the causal analysis of the development of the central nervous system into the determination of the 'ground plan' of the primary structures and the more dependent development of the 'secondary' ones (specific formations).

6. The brains of the secondary embryonic rudiments of these duplicitas are formed in all cases up to a certain level; caudally to this level the brain-parts are

normally proportioned, but the most anterior part may be reduced in size. This material can be arranged into a complete regression series, beginning with the reduction of the telencephalon and ending with a completely acephalic embryo. From this development one may conclude that the determination of the central nervous system occurs in a large number of successive zones, which are qualitatively 'equivalent', and which are determined by (qualitatively or quantitatively) different values of the organizing 'agent'.

7. There are indications that the infundibulum and hypophysis are determined by a triple contact between presumptive neural plate, prechordal plate and cephalic ectoderm after the appearance of the neural plate.

8. After special staining these incomplete nervous systems may form a very important source of data for the causal analysis of the development of tracts and nuclei in the central nervous system.

9. With the conclusions drawn from these experiments and the data given in the extensive literature on this subject, a new working hypothesis on the determination of the central nervous system is put forward. This theory involves the assumption (among others) of an equilibrium reaction between a strong mesodermal and a weak ectodermal gradient. The character of the determination process probably changes from being at first purely quantitative to become qualitative with increasing age.

10. Finally, the regional determination of the secondary rudiment in duplicitas is probably determined by a triple interaction between the induction field of the graft, the primary regional structure of the ectoderm and the regional influences of the primary rudiment.

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SEASONAL CYCLICAL CHANGES IN THE THYMUS OF THE MALLARD*

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(With Plate 5 and Four Text-figures)

In the course of an investigation into seasonal changes in the endocrine organs of wild mallards (*Anas platyrhinchas* p. (L.)), a striking seasonal cycle was found in the thymus affecting both the amount of thymus tissue and its histological structure. This appears to be the first description of changes of this nature affecting the thymus apart from a statement by Jolly (1913) in connexion with age involution of the thymus in fowls, to the effect that this tissue is much influenced by seasonal and nutritive changes. Aimé (1912) has described a seasonal regeneration of the thymus in turtles, but it will be shown below that this must be regarded as a different type of response from that found in the mallard.

MATERIAL AND METHODS

Male and female adult mallards were shot in one of the London Royal Parks at fortnightly intervals for the period May to July 1944. A second series of birds was obtained from October 1944 to January 1946. During the period November to December 1945 six adult males were killed within a period of 4 weeks in order to give an indication of the degree of individual variation in thymus weights. One male and five female immature birds were also obtained, while in July and August 1946 three additional adults were shot to amplify the data for the period of maximal thymic regeneration.

The freshly dissected lobes of thymus were weighed on a torsion balance when weighing up to 500 mg., or if heavier than this on a chemical balance. The glands were then fixed in formol acetic Muller's solution, and serial sections of part of a lobe stained in Delafield's haematoxylin and alcoholic eosin.

RESULTS

The avian thymus develops from the third and fourth branchial pouches, and in the bird just hatched appears as a long thin formation running from the level of the hyoid arch cranially to that of the eleventh segment caudally, on both sides. Where this mass is crossed by the cervical nerves it becomes constricted and finally divided into several discrete lobes (Streseman, 1934).

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* This paper is part of a thesis for which the degree of Master of Science in Physiology has been granted by the University of London.

In the mallard these lobes were found to lie in the subcutaneous fat of the neck just lateral to the jugular vein and thyroid (see Text-figs. 1, 2), a position corresponding to that described for birds in general by Streseman. The number of lobes was found to be inconstant, but five on each side was the typical finding.

Even when the number of lobes on both sides was the same, the weight of thymus tissue present in corresponding positions on the two sides was often very different. The most caudal lobe lying in the entrance to the thorax and behind the clavicle was constantly the largest.

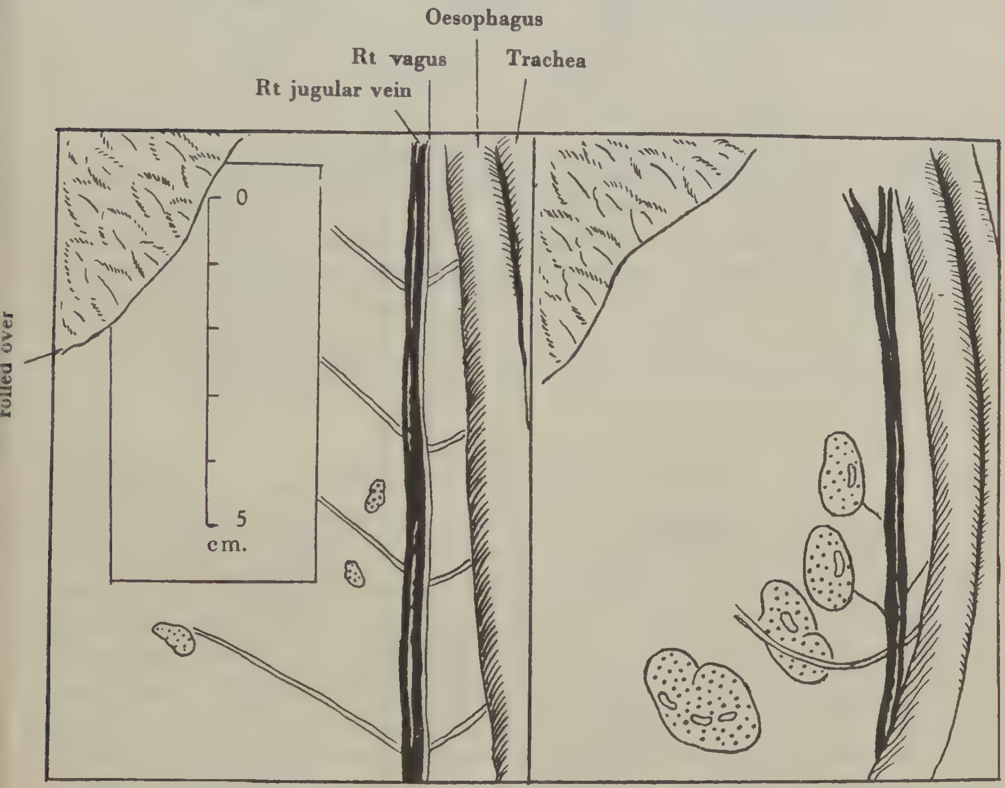


Fig. 1. Fig. 2.

Text-figs. 1, 2. Outline tracings from photographs of exposures of the right side of the neck in adult male mallards (natural size).

Text-fig. 1. From a bird killed 18 January 1946 to show the small thymus (three lobes) characteristic of the period of non-enlargement, i.e. for 10 months of the year.

Text-fig. 2. From a bird killed 31 July 1946 to show the enlarged thymus (four lobes) characteristic of the period of enlargement—July and August.

Seasonal changes in thymus weight

Table 1 gives the thymus weights of all birds examined arranged according to the time of year when they were killed. In a certain number of cases no thymus tissue could be found; this was due to the fact that, until the first birds with enlarged thymi

Table 1. *Total thymus weight of mallards at various times of the year*

Period of enlargement of thymus

Adult males		Adult females	
Date	Thymus wt. (g.)	Date	Thymus wt. (g.)
16. vii. 45	1·372	19. vii. 44	0·610
19. vii. 45	3·045	17. vii. 45	3·945
31. vii. 46	5·000	2. viii. 44	2·395
2. viii. 44	3·000	10. viii. 46	3·200
10. viii. 46	3·720		
13. viii. 45	1·625		
Immature males		**Immature females**	
Date	Thymus wt. (g.)	Date	Thymus wt. (g.)
23. ix. 45	1·237	18. vii. 45	5·216
		31. vii. 46	5·260
		20. viii. 45	3·443
		26. ix. 45	3·240
		12. xi. 45	1·300

Period of non-enlargement of thymus

Adult males		Adult females	
Date	Thymus wt. (g.)	Date	Thymus wt. (g.)
11. i. 45	0·235	18. i. 45	0·265
12. i. 46	0·305	19. ii. 46	0·155
15. ii. 45	0·178	22. ii. 45	0·240
15. iii. 45	0·147	22. iii. 45	0·062
11. iv. 45	0·135	24. iv. 45	0·034
9. v. 44	Not found	9. v. 44	Not found
24. v. 44	Not found	24. v. 44	Not found
7. vi. 44	Not found	25. v. 45	0·775
19. vi. 45	0·104	7. vi. 44	Not found
21. vi. 44	Not found	21. vi. 44	Not found
8. vii. 44	Not found	26. vi. 45	0·363
17. ix. 45	0·231	8. vii. 44	Not found
23. x. 44	0·365	30. x. 45	0·375
15. xi. 45	Not found.	18. xii. 44	0·260
20. xi. 44	0·295		
20. xi. 45	0·103		
23. xi. 45	0·191		
28. xi. 45	0·273		
29. xi. 45	0·172		
11. xii. 44	0·135		
12. xii. 45	0·279		
Mean and its S.E. for 15 observations: 0·21 ± 0·02 g.		Mean and its S.E. for 9 observations: 0·281 ± 0·073 g.	

were encountered, the precise location of the lobes was not familiar to the observer and small lobes were probably overlooked. There is, however, no doubt that in these cases the total thymus weight did not exceed 0·3 g. With this fact in mind the data from these birds have been included (but of course not used in the calculation of means), as they contribute to the determination of the onset of the period of thymic enlargement.

The table shows that during the period 16 July to 13 August adult birds have a thymus weight of the order of ten times that found at all other periods of the year. This deviation above the mean obtained for thymus weights during the rest of the year is certainly significant.

Thymus weight in relation to age and sex

During the short annual period of enlargement of the adult thymus, thymus weights of immature birds and adults are of the same order. On the other hand, at all other periods of the year immature birds have a significantly greater amount of thymus tissue than adults of either sex. The mallard, therefore, shows regression of the thymus following the attainment of sexual maturity. This relationship has been found in a number of mammalian forms and among birds, in the pigeon (several species) (Riddle & Frey, 1925), and the fowl (Greenwood, 1930).

The results available do not give evidence of a significant difference in thymus weight in the two sexes.

Histological findings

(1) *Thymus of immature birds*

The thymus lobes are divided into lobules by thin strands of connective tissue in which capillary vessels are seen. There is an obvious demarcation line between cortex and medulla. The cortex consists mainly of closely grouped cells indistinguishable from typical lymphocytes, as seen in the cervical lymph glands, with relatively few reticular or other connective tissue cells. The medulla is less cellular, and consists mainly of lymphocytes; the reticular cells are more evident than in the cortex, and relatively large islands of epithelial elements with eosinophilic cytoplasm devoid of visible cell boundaries are seen. If these epithelial islets are traced through a number of serial sections Hassal's corpuscles are to be found in them and in them only. The Hassal's corpuscles, therefore, appear to be derived from these epithelial cells. Occasionally single cells with non-granular eosinophilic cytoplasm and a pale nucleus are seen in the medulla and appear to be single cells of the islands mentioned above. Blood vessels, of the size of arterioles, are surrounded by condensations of these eosinophilic cells in a manner suggesting the outer layers of the Hassal's corpuscles.

(2) *Thymus of adult birds* (Pl. 5)

During the period July and August when large thymic lobes are found in adult birds they are indistinguishable from those of immature birds, the same lobulation and the same clear demarcation of cortex and medulla being found (Pl. 5, figs. 3 and 4).

Outside these periods when the total thymus tissue weighs about a tenth of that of immature birds, the histological appearance is also quite different. It is now quite impossible to distinguish cortical and medullary zones and all traces of lobulation are lost. The whole lobe is a uniform mass of lymphocytes with a reticular basis and the usual islands of eosinophilic epithelial syncytium and Hassal's corpuscles. Counts of the number of epithelial islands and Hassal's corpuscles found in strips of gland of uniform width were made, and these suggest that the number of these elements is relatively constant at all periods.

The thymus tissue of females killed in May and June showed an appearance not seen at other times of the year and never found in males. There was a peripheral zone of granular eosinophils, and from this zone strands of eosinophils, several cells in thickness, divided the gland into lobules, just as they are divided by connective tissue in the immature thymus or the thymus of adults in July and August. A number of these eosinophils had two nuclei and appeared to be undergoing mitotic changes. It is not possible to interpret the meaning of this eosinophil invasion.

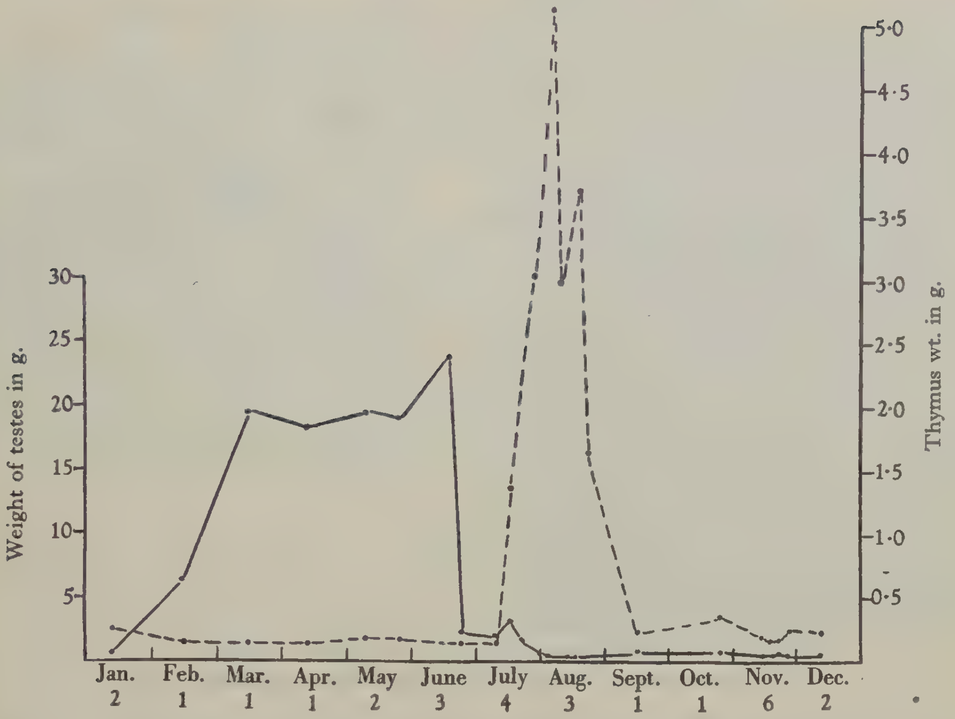
Effect of disease on thymus weight

Riddle & Frey (1925) found that in pigeons, thymus weight is readily depressed by concomitant disease in other parts of the body. This effect was also observed in two male mallards examined in this series. The thymus weight of a bird with a dermatosis of the neck was 40 mg., that of a bird with subacute pancreatitis 82 mg., showing a significant decrease below the mean weight for males of 210 ± 20 mg.

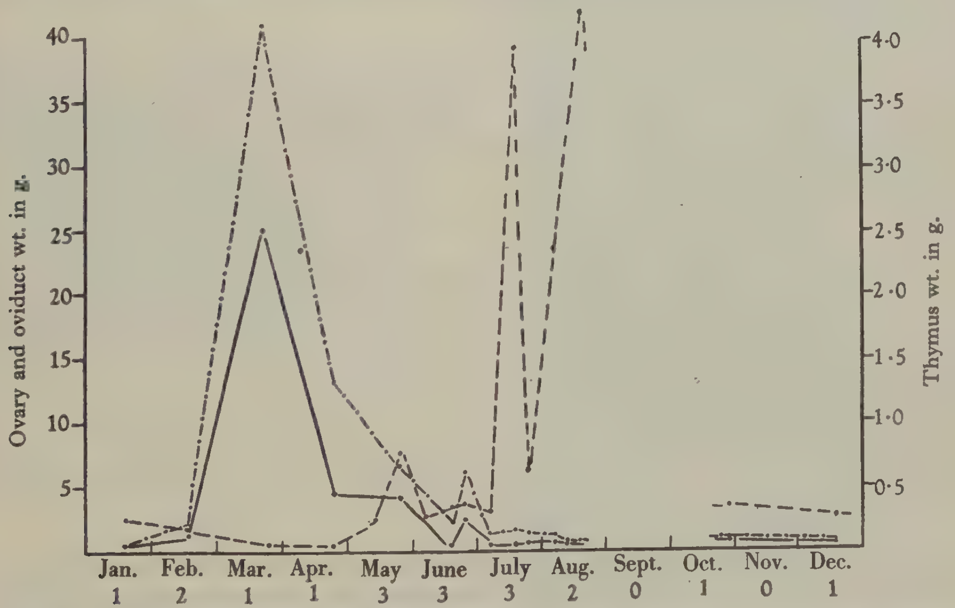
DISCUSSION

The results reported above lead to the conclusion that in the adult mallard the thymus regenerates annually for a short period of about 4 weeks following upon the end of the breeding season. It is difficult to explain the significance of this phenomenon on the basis of the established functions of the thymus, viz. formation of lymphocytes and granulocytes. Riddle's original suggestion (Riddle, 1924) that the thymus is essential for the production of the 'egg envelope' in birds has been disproved by a number of later workers including Riddle himself (Riddle & Krizenecky, 1931; Greenwood & Blythe, 1931; Morgan & Grierson, 1930; Maughan, 1938), all of whom found that thymectomy did not interfere with the laying of normal eggs. Similarly, no reactivation in the thymi of the female mallards at the period of egg laying was found in the present series.

In Text-figs. 3 and 4 thymus and gonad weights of the mallards examined are shown graphically. The period of thymus regeneration clearly occurs well after the annual period of gonadal activity is passed. The period of July and August was also the only one in which sexual behaviour was absent in these birds (sterile mating outside the breeding season occurs in the mallard in autumn and winter). Hence the period of thymic enlargement occurs at a time when it is reasonable to believe that the production of sex hormones is at a minimum. Experimental work has shown that certain sex hormones have the effect of causing thymic atrophy. Evans & Simpson (1934) obtained thymic atrophy in adult and immature white rats after the



Text-fig. 3. Seasonal weights of testes (continuous line) and thymus (interrupted line) of adult male mallards. The figures below the months indicate the number of birds examined.



Text-fig. 4. Seasonal weights of ovaries (continuous line), oviducts (· — · —) and thymus (interrupted line) of adult female mallards. The figures below the months indicate the number of birds examined.

injection of gonadotrophic extracts. The effect was lost after castration and was, therefore, mediated by the gonads. Carrière, Morel & Gineste (1937) obtained thymic atrophy in rats following injections of 'folliculin', while progesterone was ineffective. Reinhardt & Weinmann (1942) showed that in male rats castration caused an increase in thymus weight, while it was decreased by injections of testosterone.

In view of these facts it seems reasonable to attribute the seasonal thymic reactivation of the mallard to the temporary cessation of the inhibitory effect exerted by the sex hormones. It should be pointed out that there is suggestive evidence that in the human female the thymus is diminished in size during pregnancy and shows a regeneration post-partum (Anderson, 1932), a phenomenon which also suggests a relationship between thymus development and certain sex hormones.

If, as suggested, the regeneration of the thymus in the mallard is dependent on the seasonal activity of the gonads, then a thymic reactivation following the period of maximal gonadal activity may be found, not only in other birds but, perhaps, also in mammals in which reproduction is seasonal.

The changes occurring in the thymi of turtles described by Aimé (1912) differ fundamentally from those observed in the mallard as regards their relationship to the breeding season. In the turtle, thymus lobes are reduced during hibernation but show a progressive enlargement in spring and summer reaching a maximum in autumn. The regeneration of the thymus in this case evidently begins during the annual breeding season. The atrophic process in the thymus, however, is to some degree similar in turtles and the mallard, for Aimé describes a reduction of the cortical zone of the atrophied thymus recalling the complete loss of cortex in the adult mallard thymus.

SUMMARY

1. The thymi of fifty-seven adult mallards killed at regular intervals were weighed and examined histologically for seasonal changes. Six immature birds were used for comparison.

2. The thymus of immature mallards is about ten times heavier than that of adults.

3. Shortly after the end of the breeding season the thymus of both sexes shows a significant enlargement, and for a period of about 4 weeks thymus weights are of the order of ten times the average weight for other times of the year. Characteristic histological appearances are described for the two periods.

4. This seasonal regeneration of the thymus is interpreted as being due to the temporary cessation of an inhibitory effect exerted by the sex hormones.

5. In two cases of disease of other tissues the thymus was considerably below average in weight.

I am grateful to the Bailiff of Royal Parks for making the mallards used in this work available to me, and to Mr L. A. Westwood for his technical assistance. The expenses were met by a grant from the Clinical Research Committee of Guy's Hospital Medical School.

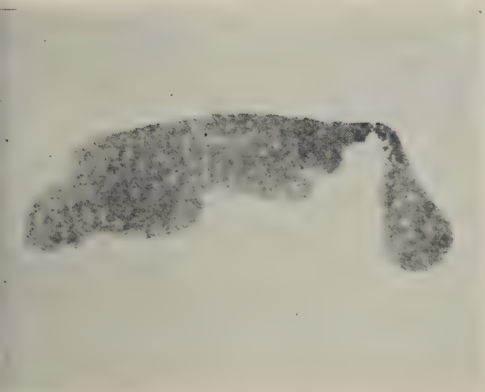


Fig. 1

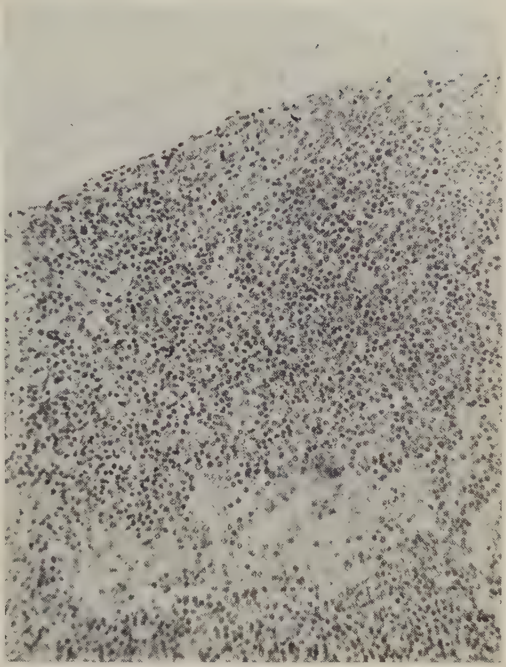


Fig. 2

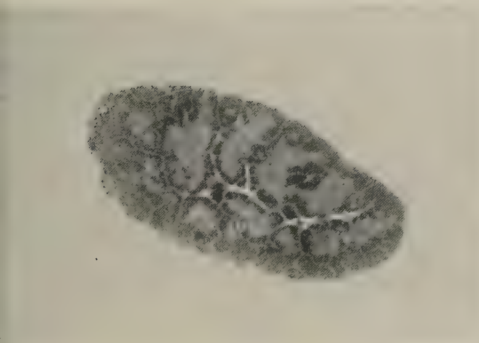


Fig. 3

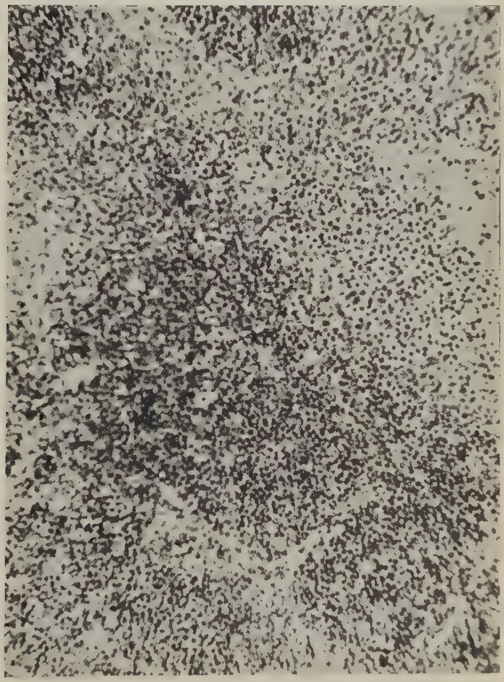


Fig. 4

ÖHN—SEASONAL CHANGES IN THE MALLARD'S THYMUS

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EXPLANATION OF PLATE 5

Fig. 1. Thymus (section) of adult male mallard killed 11 April 1945 (×7). No differentiation of cortex and medulla; no lobulation apparent.

Fig. 2. Thymus (section) of adult male mallard killed 11 April 1945 (×320). One epithelial island is shown; no cortical zone appears at edge of section.

Fig. 3. Thymus (section) of adult male mallard killed 16 July 1945 (×7) during period of thymic enlargement. Cortical and medullary zones well demarcated; lobulation well developed.

Fig. 4. Thymus of adult male mallard killed 16 July 1945 (×320) during period of thymic enlargement. Medullary area in upper right-hand corner; rest of field occupied by cortical zones of two adjacent lobules.

STUDIES ON STERILITY AND PRENATAL MORTALITY IN WILD RABBITS

PART III. THE LOSS OF OVA BEFORE IMPLANTATION

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(With Two Text-figures)

INTRODUCTION

The total loss of ova that has occurred between the time of ovulation and of examination can be estimated in pregnant animals from the discrepancy between the number of corpora lutea in the ovaries and of developing embryos. This loss will include both loss of ova through being unfertilized and loss through the subsequent death of developing embryos. Errors arise in such estimates through inaccuracies in the counts and from occasional follicles producing more than one embryo, through polyovuly, but the magnitudes of these errors in the present work on the wild rabbit have been estimated previously (Allen, Brambell & Mills, 1947).

Provided that the data are derived from animals examined at a stage of pregnancy after implantation has occurred the total loss estimated in this way can be divided into that occurring between the times of ovulation and of implantation, and that between the times of implantation and of examination, by using counts of implantation sites as well as those of corpora lutea and of surviving embryos. The excess of the number of corpora lutea over the number of implantation sites is equivalent to the loss before implantation, and the excess of the number of implantation sites over the number of surviving embryos is equivalent to the loss after implantation, subject to the qualifications mentioned above and dealt with previously (Allen *et al.* 1947). It has been shown also in the paper referred to that the loss before implantation is distributed independently of that after implantation. Therefore, it is possible, and it is convenient, to deal with them separately, and the loss after implantation will be reserved for a later paper.

Estimates of the loss before implantation derived from the difference between the numbers of corpora lutea in the ovaries and of implantation sites in the uteri are subject to two limitations. First, since they are based on counts of implantation sites, they include only litters in which some or all of the ova have survived to become implanted. Litters in which all the ova are lost before implantation are excluded. It is essential that this should be appreciated. Estimates of the loss before implantation differ from those of the loss after implantation in this respect, for the latter includes the loss of whole litters. Secondly, such estimates, since they are based on examinations made at any time after implantation, provide no information regarding the stage before implantation at which loss occurs.

The only method of determining the stage at which loss before implantation occurs and of distinguishing unfertilized ova from developing embryos that die is to recover the degenerating ova by transfusion of the Fallopian tubes or uteri. Since degenerating ova or early embryos disintegrate and disappear very rapidly the chance of recovering them in the interval between their death and disappearance is small. Moreover, for technical reasons it is difficult to recover all the ova with certainty by transfusion. They are particularly liable to be lost or overlooked when they are in the fimbria before entering the tube or are at the tubo-uterine junction, where the tube must be severed from the uterus for transfusion, since the canula is liable to be inserted past them. Thus although some information not otherwise available can be obtained from pre-implantation stages of pregnancy by transfusion of the tubo-uterine tract, this method does not provide reliable data for estimating the magnitude of the loss before implantation.

OBSERVATIONS

The distribution of the loss before implantation according to the initial size of litter

Two measurements of the embryonic mortality in a polytocous mammal such as the rabbit are available: (1) the proportion of litters showing loss, and (2) the proportion of embryos lost. The proportion of litters showing loss is related to the proportion of embryos lost, but the relationship will vary according to the distribution of the mortality. They provide, therefore, a means of analysing the mortality. The following considerations should clarify this point. Biologically it is obvious that prenatal mortality would result either from the mother being unable to bring the litter to birth or from any given embryo being non-viable: that is through failure of either the maternal or embryonic organisms. Prenatal mortality resulting from maternal failure will fall on the litters as units and consequently the proportion of litters showing loss will coincide with the proportion of ova lost. Prenatal mortality resulting from embryonic failure will fall on the embryos as units and consequently the proportion of litters showing loss will tend to vary as

$$1-q^x, \tag{i}$$

where $p+q=1$, $p=$ the proportion of ova lost and $x=$ the initial number of ova in the litter. This relation depends on the assumption that the ova lost are distributed at random in relation to the litters. If they are not so distributed a third and intermediate type of relationship between the proportion of ova lost and the proportion of litters showing loss will exist. This will be the case if the probability of loss of ova is greater in some litters than in others; for example, either through certain mothers providing an unfavourable but possible environment in which only the stronger embryos survive or through the enhanced probability of sibs inheriting the same lethal genes. The first of these three types of relationship is excluded from estimates of loss before implantation based on counts of corpora lutea and of implantation sites because none of the ova would survive to become implanted.

All the available data are contained in Table 1 in the form of a correlation table of number of ova lost with number of ova ovulated. The data are summarized according to the percentage of litters showing loss and the percentage of ova lost in the columns to the right of the correlation table. Straight regression lines have been fitted to the data. The regression for the proportion of ova lost is given by the formula

$$Y = 0{\cdot}01395x + 0{\cdot}00895. \tag{ii}$$

The regression for the proportion of litters showing loss is given by the formula

$$Y = 0{\cdot}0858x - 0{\cdot}1289. \tag{iii}$$

These regression lines, together with the observed proportions for each litter-size group from 3 to 10 inclusive, are represented graphically in Fig. 1. It will be seen that the fit is remarkably good for biological data and that the regressions are obviously significant.

On the assumption that the loss falls on the ova as units and is distributed at random amongst the litters, the proportion of litters showing loss would be derivable from formula (i), the value of q, which varies according to the initial size of litter, being obtained from equation (ii). The proportion of litters showing loss, L, should then be

$$L = 1 - (0{\cdot}99105 - 0{\cdot}01395x)^x. \tag{iv}$$

The resulting curve is indicated by the broken line in Fig. 1, and it can be seen that it exceeds appreciably the observed proportion of litters showing loss. The observed and expected numbers of litters showing no loss in the sample are shown in Table 2 and the χ^2 test is applied. Because the two values of x = the deviation sum to zero, one degree of freedom exists for each litter-size group, $n=7$, and it is clear that the divergence of the observed from the expected values is significant. Thus fewer litters have suffered loss than would be the case if the loss were distributed at random amongst the litters or, otherwise expressed, there is a tendency for the loss to be concentrated in some of the litters. That this is in fact true can be shown more clearly by comparing the observed number of litters showing a loss of 0, 1, 2, 3 or more ova each respectively, with the expectation derived from the expansion of the binomial, as is done in Table 3. It is clear that the number of litters showing a loss of three or more ova each is substantially in excess of the expectation, and that testing by means of χ^2 the observed distribution differs significantly from the expectation.

The possibility remains that although the loss tends to be concentrated in certain litters it is distributed at random amongst those litters that do suffer loss. This can be tested by calculating the expected distribution for values of p derived from equation (iii). The equation becomes

$$p = 1 - \sqrt[x]{(1{\cdot}1289 - 0{\cdot}0858x)}. \tag{v}$$

This amounts to calculating the random distribution amongst a known number of litters showing loss. The observed and expected values are compared in Table 4, and it is clear that there is significant excess of litters showing a loss of two or more ova over the expectation. It must be concluded that the sample of litters showing loss is not homogenous, but that the probability of loss of ova is greater in some of them than in others.

Table 1. *Loss of ova before implantation. All data 7–32 days pregnant,* 1941–5

No. of ova ovulated	No. of ova lost									Litters			Ova		
	0	1	2	3	4	5	6	7	8	Total	No. showing loss	% showing loss	Total	No. lost	% lost
12	—	—	—	—	—	—	—	—	1	1	1	—	12	8	—
11	1	1	—	—	—	1	—	—	—	3	2	—	33	6	—
10	3	4	2	2	—	—	1	—	—	12	9	75·0	120	20	16·7
9	23	19	15	2	1	4	—	—	—	64	41	64·1	576	79	13·7
8	73	59	32	8	7	1	—	—	—	180	107	59·4	1440	180	12·5
7	208	128	50	9	4	2	1	—	—	402	194	48·3	2814	287	10·2
6	355	144	38	11	5	1	—	—	—	554	199	35·9	3324	278	8·4
5	352	105	23	7	6	—	—	—	—	493	141	28·6	2465	196	8·0
4	276	63	17	6	—	—	—	—	—	362	86	23·8	1448	115	7·9
3	89	13	3	—	—	—	—	—	—	105	16	15·2	315	19	6·0
2	1	—	—	—	—	—	—	—	—	1	0	—	2	0	—
1	2	—	—	—	—	—	—	—	—	2	0	—	2	0	—
Total	1383	536	180	45	23	9	2	—	1	2179	796	36·5	12551	1188	9·5

Table 2

No. of ova ovulated	Litters showing no loss				Litters showing loss			
	Observed	Expected $=m$	Divergence $=x$	x^2/m	Observed	Expected $=m$	Divergence $=x$	x^2/m
>9	27	20·4	+ 6·6	2·13	53	59·6	− 6·6	0·74
8	73	64·4	+ 8·6	1·15	107	115·6	− 8·6	0·63
7	208	182·8	+25·2	3·47	194	219·5	−25·5	2·96
6	355	308·8	+46·2	6·91	199	244·6	−45·6	8·50
5	352	327·2	+24·8	1·88	141	165·7	−24·7	3·69
4	276	276·9	− 0·9	0·00	86	85·0	+ 1·0	0·01
<3	92	92·7	− 0·7	0·01	16	15·3	+ 0·7	0·04
Total	1383	1273·2	+109·8	15·55	796	905·3	−109·3	16·57

$\chi^2=32{\cdot}12,\quad n=7,\quad P<0{\cdot}01.$

Table 3

	No. of ova lost				
	0	1	2	3	4+
Observed	1383	536	180	45	35
Expected	1273·2	673·1	189·0	36·9	6·3
Divergence	+109·8	−137·1	−9·0	+8·1	+28·7
x^2/m	9·47	27·92	0·43	1·78	130·74

$n=4,\quad \chi^2=170{\cdot}34.$

Table 4

	No. of ova lost		
	1	2	3+
Observed	536	180	80
Expected	624·9	145·1	26·0
Divergence	−88·9	+34·9	+54·0
x^2/m	12·65	8·39	112·15

$n=2,\quad \chi^2=133{\cdot}19.$

The distribution of the loss before implantation between the two uteri of each pair

The problem of whether the loss of two or more ova is distributed at random between the two uteri of each pair or tends to be concentrated on one side requires investigation. The first alternative might be expected to result from systemic deficiency on the part of the mother, for example, endocrine or nutritional deficiency, or from genetic factors affecting the viability of the embryos. The second alternative would result from morphological abnormalities or pathological lesions obstructing the lumen of the Fallopian tube or uterus on one side, and thus tending to prevent either fertilization by obstructing the passage of spermatozoa, or the ova reaching the uterus. Clearly only litters that had suffered a loss before implantation of two

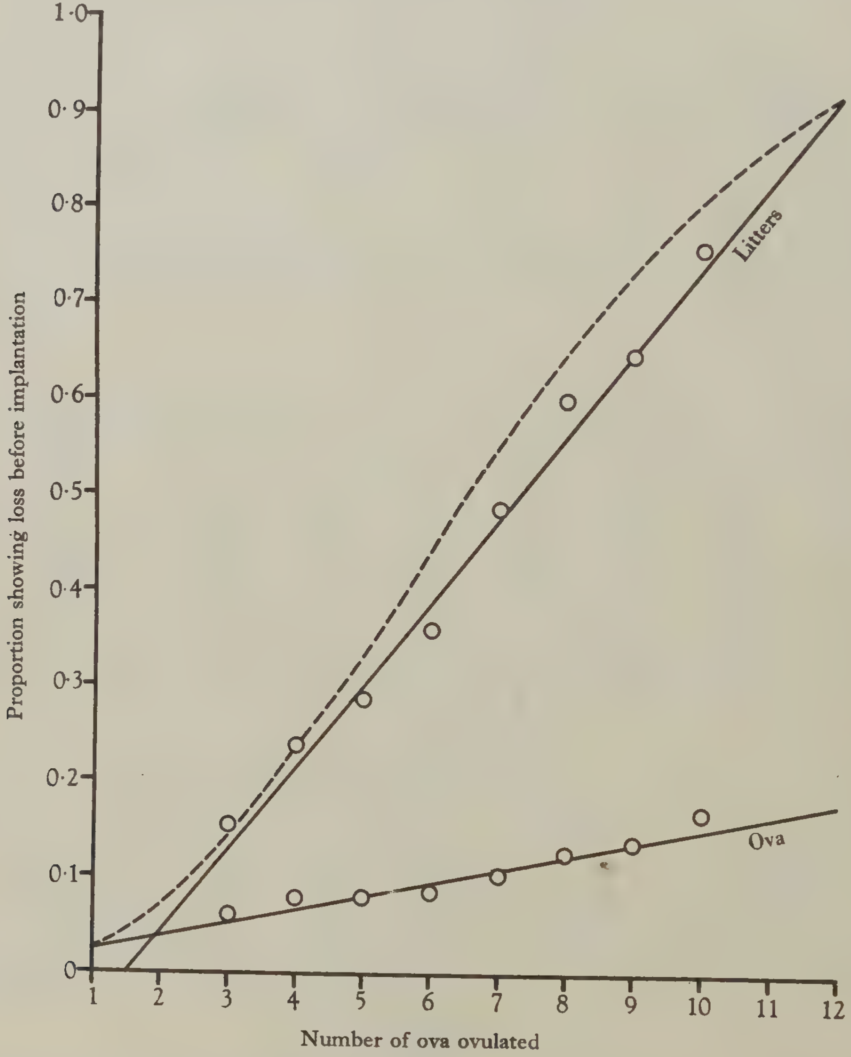

Fig. 1.

or more ova could be employed. The distribution of the difference between the loss on one side and that on the other side in 254 such litters, according to the initial size of litter, is given in Table 5. Six litters with a loss of two or more ova before implantation that are included in Table 1 were not available, either because transmigration of ova had occurred or because the distribution of the loss between the two sides was not recorded. The expected random distribution of the loss can be calculated for each litter-size group provided that (i) the data for the initial distribution of ova between the two sides, as shown in the number of corpora lutea in the two ovaries (Table 6), and (ii) the data for the number of litters showing a loss of 2, 3, 4, etc. (Table 7), are known. Suppose that x is the initial size of litter, x' is the number of ova lost, x'' is the number of ova surviving, and a is the number of corpora lutea in the left ovary. Then the probability that n ova should be lost in the left uterus and hence $x'-n$ in the right uterus is

$$T'' \times T'/T, \tag{vi}$$

where T is the $(a+1)$th term in the expansion of $(1+1)^x$, T' is the $(x'-n+1)$th term in the expansion of $(1+1)^{x'}$, and T'' is the $(a-n+1)$th term in the expansion of $(1+1)^{x''}$. The expectation of any given combination of x' and n can be obtained by multiplying the probability by the proportion of litters of initial size x which show a loss of x' ova, and by the number of these litters which are distributed with $(x-a)$ corpora lutea in the right ovary.

The expectation, derived in this way, for the data in Table 5 is given in the third column from the right. Testing by means of $\chi^2 = 15{\cdot}42$ and $n=3$ it is clear that the divergence of the observed from the expected values is highly significant, since $P<0{\cdot}01$. The divergence of the observed from the expected values is symmetrical and shows a tendency for the loss of two or more ova to be concentrated in one uterus of a pair. If the lumen of the uterus or Fallopian tube was completely obstructed on one side but not on the other, then all the ova on that side would be lost before implantation. It appeared probable that this might account for some or all of the tendency towards concentration of the loss in one uterus. This was tested by determining the numbers of uterine horns, treated independently of each other, in which either some of the ova survived or all were lost. The data of animals which had lost only a single ovum were relevant and were added to those which had lost two or more. The expected distribution for each of these groups was calculated in the same way as that for Table 5. The results are given in Table 8, and it can be seen therefrom that the number of uteri in which all the ova were lost exceeds the expected number by 18·06. Testing by means of $\chi^2 = 23{\cdot}65$, $n=7$, it is clear that as $P<0{\cdot}01$ this difference is highly significant. Support for the suggestion that this excess is due to unilateral obstruction of the uterine tract is provided by three animals included in Table 5 in which obstruction of the uterine tract had been observed macroscopically at the time of dissection. The Fallopian tube was observed to end blindly in one of these, in another there were extensive adhesions, and in the third one uterus was rudimentary. Similar abnormalities were observed on dissection in a number of other rabbits which do not fall within the group under consideration because they were non-pregnant, or had suffered a total

Table 5. *Distribution of loss of two or more ova before implantation between the two uteri according to initial size of litter*

Difference of loss between the two uteri	Initial size of litter										Observed total	Expected $=m$	Divergence $=x$	x^2/m
	3	4	5	6	7	8	9	10	11	12				
6	—	—	—	—	—	—	—	—	—	—	—	0·09	+15·12	12·11
5	—	—	—	—	1	—	—	—	1	—	2	0·52		
4	—	—	1	1	3	2	1	—	—	—	8	3·89		
3	—	4	2	4	4	4	4	2	—	—	24	14·38		
2	2	11	19	17	25	19	9	1	—	—	103	103·15	− 0·15	0·00
1	—	2	5	8	6	5	2	—	—	—	28	39·10	−11·10	3·15
0	1	6	9	23	26	15	6	2	—	1	89	92·87	− 3·87	0·16
Total	3	23	36	53	65	45	22	5	1	1	254	254·00	0·00	15·42

Table 6. *Distribution of corpora lutea between the two ovaries in litters losing two or more ova before implantation*

No. of corpora lutea in right ovary	Initial size of litter										Total
	3	4	5	6	7	8	9	10	11	12	
9+	—	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	—	1	—	—	—	—	1
7	—	—	—	—	3	2	—	—	—	1	6
6	—	—	—	2	6	6	4	2	1	—	21
5	—	—	1	8	10	13	4	1	—	—	37
4	—	—	3	13	19	10	8	1	—	—	54
3	1	7	10	14	12	6	6	1	—	—	57
2	1	11	9	9	9	5	—	—	—	—	44
1	—	5	12	7	6	2	—	—	—	—	32
0	1	—	1	—	—	—	—	—	—	—	2
Total	3	23	36	53	65	45	22	5	1	1	254

Table 7. *Distribution of loss of two or more ova before implantation according to initial litter size*

No. of ova lost	Initial size of litter										Total
	3	4	5	6	7	8	9	10	11	12	
8	—	—	—	—	—	—	—	—	—	1	1
7	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	1	—	—	1	—	—	2
5	—	—	—	1	2	1	4	—	1	—	9
4	—	—	6	5	4	6	1	—	—	—	22
3	—	6	7	11	9	8	2	2	—	—	45
2	3	17	23	36	49	30	15	2	—	—	175
Total	3	23	36	53	65	45	22	5	1	1	254

loss of one or none, or were less than 7 days pregnant. Assuming that the whole of the excess over the expectation of eighteen animals which have lost all the ova in one horn is due to occlusion of the reproductive tract on one side it is clear that this number out of a total of 2073 represents only 1·8%. (106 litters are included in Table 1 which have been excluded from these calculations either because the distribution of embryos between the two uteri was not recorded or was complicated by the occurrence of transmigration of ova or polyovuly.) This is obviously far too small a proportion to account for more than a small fraction of the tendency towards multiple loss of ova in a litter in excess of the random expectation which is shown in Table 1.

Table 8. *Distribution of loss of all the ova from one uterine horn in litters that have lost some ova before implantation*

Initial no. of ova in uterine horn	Uterine horns with some ova surviving				Uterine horns having lost all ova			
	Observed	Expected	Divergence	χ^2	Observed	Expected	Divergence	χ^2
8	3	3·00	+0·01	0·00	—	0·00	+12·32	22·72
7	20	19·99			—	0·01		
6	76	75·97	+0·03	0·00	—	0·03		
5	190	190·73	−0·73	0·00	1	0·27		
4	297	301·62	−4·62	0·07	6	1·38		
3	397	404·01	−7·01	0·12	12	4·99		
2	294	296·76	−2·76	0·03	19	16·24	+2·76	0·47
1	125	127·98	−2·98	0·07	54	51·02	+2·98	0·17
Total	1402	1420·06	−18·06	0·29	92	73·94	+18·06	23·36

$\chi^2 = 23·65, \quad n = 7, \quad P = < 0·01.$

Table 9. *Occurrence of loss in one, both or neither of the uterine horns*

Distribution of loss between the two uteri	Observed	Method 1			Method 2		
		Expected $= m$	Divergence $= x$	x^2/m	Expected $= m$	Divergence $= x$	x^2/m
No loss	1305	1268·3	+36·7	1·06	—	—	—
Loss in right uterus only	324	360·7	−36·7	3·73	312·9	+11·1	0·39
Loss in left uterus only	309	345·7	−36·7	3·90	307·2	+ 1·8	0·01
Loss in both uteri	135	98·3	+36·7	13·70	147·9	−12·9	1·13
Total	2073	2073·0	0·0	$\chi^2 = 22·39$	768·0	0·0	$\chi^2 = 1·53$

It is appropriate, at this point, to inquire whether the occurrence of loss in one uterine horn affects the probability of loss in the other. The data for the 2073 litters available, referred to in the last paragraph, are summarized in Table 9.

Treating the uterine horns as units, on the simple alternative of loss or no-loss, irrespective of the number of ova lost, the number of litters expected to show loss in both horns on the assumption that loss in one horn is independent of that in the other can be calculated simply from the total number of litters, the total number with loss on the right and the total number with loss on the left. The expectations for the other groups follow. The results, together with the calculation of $\chi^2 = 22\cdot39$, are given in Method 1 in Table 9. Since $n = 1$, it is clear that the observed distribution differs very significantly from the expectation, P being much less than $0\cdot01$. The excess of litters suffering loss in both horns could be accounted for by the tendency, already demonstrated and referred to above, to suffer multiple loss in excess of the random expectation. Clearly a litter suffering a loss of five is much more likely to have lost some from each uterus than one losing only two. Consequently the expectation was calculated for the known distribution of ova between the two horns and the known incidence of loss of one, two, three or more ova for each litter-size group, from the formula (vi) given. The results and the calculation of $\chi^2 = 1\cdot53$ are given under Method 2 in Table 9. Since $n = 2$, P is between $0\cdot3$ and $0\cdot5$, and the expectation satisfactorily fits the data. It may be concluded that the occurrence of loss in one horn does not either increase or decrease the probability of loss in the other horn, when the amount of loss and the distribution of the embryos is taken into account. This means that the factors which produce the mortality tend to operate on the two uterine horns equally, not on one horn independently of the other, and that the number of horns which are sterile through occlusion of the lumen, or some such cause, is too small to affect the significance of the fit when they are included in the whole sample. Thus the tendency towards multiple loss in some litters, referred to on p. 194, cannot be accounted for by factors, such as occlusion, operating on one horn independently of the other, but must affect the litter as a whole.

The incidence of the loss of whole litters before implantation

Ovulation in the rabbit is dependent on copulation, so that all rabbits with corpora lutea must have copulated. Ovulation occurs some 12 hr. after copulation and the ova are fertilized in the upper parts of the Fallopian tubes soon afterwards. Animals with recent, that is, developing, corpora lutea in the ovaries but without implanted embryos in the uteri must belong to one of the four following categories: (*a*) those taken between the times of ovulation and fertilization, a brief period of only a few hours' duration; (*b*) those in which fertilization has failed to occur through incapacity either of the ova or of the spermatozoa or through occlusion of the uteri or Fallopian tubes preventing access of the one to the other; (*c*) those in which all the fertilized ova have already died at some stage before implantation; (*d*) those with developing embryos. Animals with old, that is, retrogressing, corpora lutea in the ovaries but without implanted living or dead embryos in the uteri must belong to one of the three following categories: (i) those in late stages of pseudopregnancy in which the ova either were not fertilized [(*b*) above], or (ii) died before implantation [(*c*) above], or (iii) those which have recently been pregnant and given birth to a litter at full term or lost it by reabsorption or abortion. Those in the last category (iii) can be recognized, as a rule, by the presence of placental sites in the uteri as well as of old corpora lutea in the ovaries, since the placental sites tend to remain recognizable macroscopically for as long as the corresponding corpora lutea.

Those animals which have corpora lutea in the ovaries but which have not implanted embryos, living or dead, nor in which living unfertilized ova (*a*) nor embryos at stages of development prior to implantation (*d*) were recovered by transfusion, nor were recently pregnant (iii) will include all those that are pseudopregnant (*b* and *c*). They will also include those animals in which unfertilized ova (*a*) or early embryos (*d*) were not recovered through failure of the transfusion technique. The amount of loss of whole litters, either through all the ova being unfertilized or through the death of all the embryos before implantation, can be estimated from the numbers in this group. Such an estimate will be an overestimate, since it will include those in which developing ova were present but none was recovered. No data are available for series 2, since no attempt was made to recover unimplanted embryos in this series (Allen *et al.* 1947). A total of sixty-two animals in the other series were obtained which were probably or possibly pseudopregnant, as compared to 820 animals in which developing embryos 0–7 days post-coitum of age were recovered. Since pseudopregnancy lasts approximately 16 days this represents a proportion of 62 pseudopregnancies to every 1640 pregnancies, or 3·64% of animals ovulating. The remains of degenerating ova were recovered in seventeen of these animals, or 1·00% of animals ovulating, and although all the ova were accounted for in only five of these, this figure may be taken as a minimum estimate of the loss. It is not practicable to divide this total loss of 1·00–3·64% of litters before implantation into that due to failure of fertilization and that due to subsequent mortality.

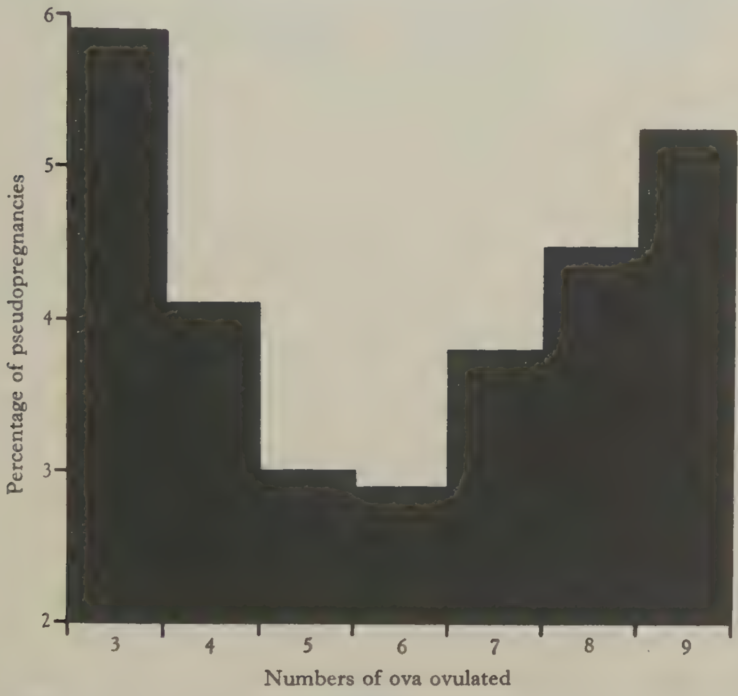

Fig. 2.

However, both uteri were observed to be obstructed in three animals and one uterus in a fourth, so that obstruction of the female tract is an appreciable factor in preventing fertilization.

The distribution of these presumed pseudopregnant animals in relation to the initial size of litter is shown in Table 10, together with the distribution of pregnancies 0–7 days post-coitum. The percentage of all animals ovulating which are presumed to be pseudopregnant is shown also. It can be seen that the percentage of pseudopregnancies is at a minimum for the most frequent litter sizes of five and six and rises uniformly towards maxima at both extremes of litter size.

Table 10. *Distribution of pseudopregnancies according to the initial size of litter*

No. of ova ovulated	No. of pregnancies 0–7 days post-coitum	No. of pseudo-pregnancies	% of pseudo-pregnancies
10+	4	0	—
9	18	2	5·3
8	64	6	4·5
7	152	12	3·8
6	221	13	2·9
5	177	11	3·0
4	130	11	4·1
3	48	6	5·9
2	5	0	—
1	1	1	—
Total	820	62	

Table 11. *Distribution of pseudopregnancies for successive 4-week periods*

Weeks	No. of pseudopregnancies	%
1–4	8	8·5
5–8	7	2·9
9–12	16	3·4
13–16	14	3·3
17–20	7	2·1
21–24	8	8·9
25–28	2	12·5

The distribution of these pseudopregnant animals throughout the breeding season is interesting also. The data for successive 4-weekly periods together with the percentages of all animals ovulating, which they represent, calculated as above, are given in Table 11. It will be seen that the percentage is higher at the beginning and at the end of the breeding season than it is during the height of the season. It might have been expected that it would rise continuously as the season progressed from an initial low value, while, in fact, it reaches a minimum in the 17–20 weeks period, after the peak of the season has been passed.

The distribution of the loss before implantation according to the body weight

The cleaned body weight is related both to the age of the animal and to its condition. Thus light animals are mainly young ones but include some old ones in poor condition. Very heavy ones are old ones in good condition. The relation of loss before implantation to body weight might be expected to be of interest in consequence. Its significance is complicated by the direct relation of initial size of litter to body weight, so that the larger animals tend to have larger litters and hence to suffer a proportionately heavier mortality on this account alone. The data for each 100 g. weight group are given in Table 12, the expectations for number of ova lost and for number of litters showing loss being calculated from the regressions (ii) and (iii). The sum of x^2/m for the separate groups gives a value of $\chi^2=8{\cdot}01$, which, for $n=6$, corresponds to $P=0{\cdot}3$–$0{\cdot}2$, so that there is no significant deviation of the observed from the expected values group by group. All those 1300 g. and over, however, show less loss, and those 1299 g. and under more loss than expected, and the values of $\chi^2=4{\cdot}60$, $n=1$, $P=0{\cdot}05$–$0{\cdot}02$ for these two groups show that the deviation is significant. The values of $\chi^2=30{\cdot}03$, $n=6$, $P=<0{\cdot}01$ for the number of ova lost show that the deviation here is clearly significant for the sample as a whole, as is also that for the individual groups 700–999 and 1400–1499 g. It may be concluded, therefore, that animals above the average body weight suffer less loss before implantation in litters that survive than those below the average body weight, as measured both by the number of litters affected and by the number of ova lost.

Seasonal variations in the loss before implantation

The distribution of the loss before implantation for successive 4-weekly periods of the year, beginning from the first Sunday in January, are given in Table 13. The expectations are calculated in the same manner as the last section. Testing by means of χ^2 it is clear that the observed do not differ significantly from the expected values in any case, either for litters or ova. It must be concluded that there is no detectable seasonal variation in the incidence of prenatal mortality occurring before implantation in litters that survive.

Distribution of loss before implantation according to whether milk is present or not in the mammary glands

The data for loss before implantation in those animals which contained milk in the mammary glands and those which did not are given in Tables 14 and 15 for comparison. Animals over 28 days pregnant were excluded from both groups, since milk appears in the mammary glands at this stage of gestation irrespective of whether the animal was previously pregnant or pseudopregnant. The large group of animals with milk will include all those which had recently stopped lactating but in which the milk had not yet entirely disappeared from the ducts, those which were recently pseudopregnant and those which had lost a litter recently without suckling it, as well as those which were actually suckling a litter. The much smaller group of those without milk will include non-parous animals pregnant for the first time and also parous animals beginning to breed at the beginning of the season after a period of anoestrus. The percentages of ova lost for each litter-size group, except for small litters of 3 and 4, are consistently lower for those with milk than for those without. It has not been found practicable to apply an entirely satisfactory statistical test of the significance of the difference between the proportion of ova lost because of the differences in the distribution of litter sizes between the two groups, but inspection suggests that it is significant.

Table 12. *Distribution of loss before implantation according to body weight*

Body weight groups (g.)	Initial size of litters													Litters					Ova				
	1	2	3	4	5	6	7	8	9	10	11	12	Total	Observed showing loss	Expected showing loss $=m$	Divergence $=x$	x^2/m		Total	Observed lost	Expected lost $=m$	Divergence $=x$	x^2/m
1700–1799	—	—	—	—	—	—	—	—	1	—	—	—	1	1	33·48	− 3·48	0·36	2·70	9	2	55·10	− 6·10	0·67
1600–1699	—	—	—	—	1	1	1	4	1	1	—	—	9	4					69	12			
1500–1599	—	—	1	2	5	19	12	15	5	2	—	—	61	25					419	35			
1400–1499	—	—	1	22	32	47	57	32	22	1	—	—	214	83	92·19	− 9·19	0·92		1396	121	146·08	−25·08	4·31
1300–1399	—	—	16	59	92	150	118	57	16	6	1	—	515	186	203·10	−17·10	1·44		3141	267	310·39	−43·39	6·07
1200–1299	—	—	29	103	145	162	117	44	12	2	1	—	615	227	221·51	+ 5·49	0·14	1·90	3506	337	327·11	+ 9·89	0·30
1100–1199	—	—	25	77	138	133	76	18	6	—	—	1	474	167	163·11	+ 3·89	0·09		2613	258	235·44	+22·56	2·16
1000–1099	—	1	21	63	65	36	20	7	1	—	1	—	215	80	64·43	+15·57	3·76		1074	120	90·01	+29·99	9·99
900– 999	1	—	9	26	13	6	1	3	—	—	—	—	59	18	18·14	+ 4·86	1·30		264	29	23·59	+12·41	6·53
800– 899	1	—	2	8	2	—	—	—	—	—	—	—	13	2					49	3			
700– 799	—	—	1	2	—	—	—	—	—	—	—	—	3	3					11	4			
Total	2	1	105	362	493	554	402	180	64	12	3	1	2179	796	795·96	+ 0·04	8·01	4·60	12551	1188	1187·72	+ 0·28	30·03

$\chi^2=8·01$, $n=6$, $P=0·30-0·20$. $\chi^2=4·60$, $n=1$, $P=0·05-0·02$. $\chi^2=30·03$, $n=6$, $P=<0·01$.

Table 13. *Distribution of loss before implantation for successive 4-week periods*

Periods in weeks	Initial size of litters													Litters				Ova				
	1	2	3	4	5	6	7	8	9	10	11	12	Total	Observed showing loss	Expected showing loss $=m$	Divergence $=x$	x^2/m	Total	Observed lost	Expected lost $=m$	Divergence $=x$	x^2/m
1– 4	—	—	18	38	23	7	—	2	—	—	—	—	88	21	21·2	−0·2	0·02	379	25	27·5	− 2·5	0·23
5– 8	—	—	31	119	88	45	17	5	1	—	—	—	306	78	84·7	−6·7	0·53	1447	112	113·9	− 1·9	0·03
9–12	—	—	31	114	145	134	64	26	4	—	—	—	518	165	170·9	−5·9	0·20	2770	243	244·7	− 1·7	0·01
13–16	—	1	8	48	136	191	148	62	21	3	2	1	621	254	248·5	+5·5	0·12	3829	377	379·2	− 2·2	0·01
17–20	1	—	6	20	61	146	147	71	36	9	—	—	497	217	218·3	−1·3	0·01	3291	342	346·2	− 4·2	0·05
21–24	1	—	4	12	31	29	25	13	2	—	1	—	118	54	44·7	+9·3	1·94	698	78	67·4	+10·6	1·67
25–52	—	—	7	11	9	2	1	1	—	—	—	—	31	7	7·8	−0·8	0·08	137	11	10·3	+ 0·7	0·05
Total	2	1	105	362	493	554	402	180	64	12	3	1	2179	796	796·1	−0·1	2·90	12551	1188	1189·2	− 1·2	2·05

$\chi^2=2·90$, $n=6$, $P=0·9-0·8$. $\chi^2=2·05$, $n=6$, $P=0·95-0·9$.

Table 14. *Animals with milk in the mammary glands*

No. of ova ovulated	No. of ova lost									Litters			Ova		
	0	1	2	3	4	5	6	7	8	Total	No. showing loss	% showing loss	Total	No. lost	% lost
12	—	—	—	—	—	—	—	—	1	1	1	—	12	8	—
11	1	1	—	—	—	—	—	—	—	2	1	—	22	1	—
10	3	4	2	1	—	—	1	—	—	11	8	72·7	110	17	15·5
9	22	19	15	1	1	4	—	—	—	62	40	64·5	558	76	13·6
8	70	56	28	8	6	1	—	—	—	169	99	58·6	1352	165	12·2
7	193	120	43	8	4	1	1	—	—	370	177	47·8	2590	257	9·9
6	317	122	28	10	3	—	—	—	—	480	163	34·0	2880	220	7·6
5	269	65	13	3	4	—	—	—	—	354	85	24·0	1770	116	6·6
4	90	27	3	2	—	—	—	—	—	122	32	26·2	488	39	8·0
3	10	4	—	—	—	—	—	—	—	14	4	28·6	42	4	9·5
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total	975	418	132	33	18	6	2	—	1	1585	610	38·5	9824	903	9·2

Table 15. *Animals with no milk in the mammary glands*

No. of ova ovulated	No. of ova lost									Litters			Ova		
	0	1	2	3	4	5	6	7	8	Total	No. showing loss	% showing loss	Total	No. lost	% lost
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	1	—	—	—	1	1	—	11	5	—
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	2	1	1	—	1	—	—	—	—	5	3	60·0	40	7	17·5
7	3	2	2	—	—	1	—	—	—	8	5	62·5	56	11	19·6
6	19	5	3	—	1	1	—	—	—	29	10	34·5	174	20	11·5
5	51	24	7	3	—	—	—	—	—	85	34	40·0	425	47	11·1
4	152	29	10	2	—	—	—	—	—	193	41	21·2	772	55	7·1
3	70	6	3	—	—	—	—	—	—	79	9	11·4	237	12	5·1
2	1	—	—	—	—	—	—	—	—	1	0	—	2	0	—
1	2	—	—	—	—	—	—	—	—	2	0	—	2	0	—
Total	300	67	26	5	2	3	—	—	—	403	103	25·6	1719	157	9·1

The loss before implantation in each series

The data for each series are given in Table 16. The various series, particulars of which have been given previously (Allen *et al.* 1947), are not strictly comparable since series 01 was confined to the beginning of the breeding season, series 0 and series 7 and 8 to the end of the breeding season, and series 5 and 6 to its height. These differences and the differences in the distribution of initial litter sizes invalidate strict statistical comparison. Apart from series 01, which is so small as to be of doubtful value, the variation in the proportion of ova lost from 8·1 to 11·9% is small. The variation in the proportion of litters showing loss from 30·8 to 41·6% is larger, but is accounted for in part by differences in initial litter sizes. The

Table 16. *Distribution of loss before implantation for each series*

Series	Initial size of litters													Litters				Ova				
	1	2	3	4	5	6	7	8	9	10	11	12	Total	Observed showing loss	Observed % showing loss	Expected % showing loss	Deviation	Total	Observed lost	Observed % lost	Expected % showing loss	Deviation
01	—	—	17	32	22	12	4	1	—	—	—	—	88	17	19·3	25·9	−6·6	397	19	4·8	7·6	−2·8
0	—	1	10	13	28	28	18	3	—	—	—	—	101	35	34·7	33·1	+1·6	542	49	9·0	8·8	+0·2
1	1	—	12	50	91	88	51	25	5	—	—	—	323	125	38·7	35·2	+3·5	1822	198	10·9	9·2	+1·7
2	—	—	6	29	41	26	15	5	1	—	—	—	123	40	32·5	32·4	+0·1	649	59	9·1	8·9	+0·2
3	—	—	17	68	59	65	56	30	12	2	2	—	311	118	37·9	36·5	+1·4	1790	187	10·4	9·6	+0·8
4	1	—	18	51	79	91	46	12	4	—	—	—	302	123	40·7	34·1	+6·6	1654	196	11·9	9·0	+2·9
5, 6	—	—	12	56	93	98	59	20	3	—	—	—	341	105	30·8	35·2	−4·4	1913	155	8·1	9·1	−1·0
7, 8	—	—	—	9	35	96	121	70	39	10	1	1	382	159	41·6	47·0	−5·4	2668	216	8·1	11·0	−2·9
9	—	—	13	54	45	50	32	14	—	—	—	—	208	74	35·6	33·1	+2·5	1116	109	9·8	8·9	+0·9
Total	2	1	105	362	493	554	402	180	64	12	3	1	2179	796	36·5	36·5	−0·7	12551	1188	9·5	9·5	0·0

expected percentage of litters showing loss, calculated from the actual distribution of initial litter sizes for each series, from regression (iii), is given, and it can be seen that the observed does not differ from the expected value by more than ±6·6% in any series.

DISCUSSION

Critical examination of the problem of estimating the loss of ova before implantation in a polytocous mammal shows that two different methods, each dealing with a part only of the total loss, must be employed. This is because of the nature of the data available on which to base such estimates.

One method is based on the comparison of the number of corpora lutea in the ovaries with the number of implantation sites in the uteri. Since the data for this method of estimation are provided only by animals in which some, at least, of the embryos have survived implantation they can give no information regarding the amount of loss of whole litters before implantation. This method provides an estimate of the amount of loss of ova in litters that survive implantation. Since it is based on the one hand on corpora lutea, not on fertilized or developing ova, it will include the loss of ova through being unfertilized, as well as through death of developing embryos between the times of fertilization and implantation, in litters that survive to become implanted. Since it is based exclusively on litters that have survived implantation, that is, on litters that have already suffered the whole of this loss, it will provide a direct measure of the total loss in these litters between ovulation and implantation. It is not based on litters in which the embryos are not yet implanted and which, therefore, are still subject to loss before implantation. This is an important distinction and one in which estimates of loss before implantation and estimates of loss after implantation differ. This method is subject to errors in the counts of corpora lutea and of implantation sites as well as to those arising from the liberation of more than one ovum from a single follicle (Allen *et al.* 1947).

The other method is based on the number of animals which are pseudopregnant, that is, on the number of animals which have corpora lutea in the ovaries but which neither are pregnant nor have recent implantation nor placental sites in the uteri. These therefore must have lost all the ova in the litter between the time of ovulation and implantation. This method will provide a direct measure of the total number of litters in which none of the ova survives implantation. It is subject to error arising through technical failure to recover any developing embryos in early pregnancies, resulting in these being identified as pseudopregnancies. It will include the loss of litters through all the ova being unfertilized as well as the loss through all the embryos dying between fertilization and implantation.

Estimates derived from these two methods are complementary and mutually exclusive. Both together provide an estimate of the total loss between ovulation and implantation. Both include the loss through non-fertilization as well as the loss through mortality. Neither provides a means of distinguishing between the losses through non-fertilization and mortality of fertilized ova. The one deals only with the loss of some ova in surviving litters, the other only with the loss of whole litters.

The mean loss of ova in litters that are lost before implantation is not more than 3·64% nor less than 1·00%. The mean loss of ova in litters that survive implantation is 9·5%. Thus the mean loss before implantation of all ova ovulated is between 10·2 and 13·0%. This appears a heavy rate of wastage for a species in which ova are only ovulated after copulation and in which, therefore, failure to mate cannot account for loss.

It has been shown that if the mortality falls on the embryos as independent units and is distributed at random amongst them, then the proportion of litters suffering loss will vary inversely as the complement of the proportion of embryos lost raised to the power of the number of embryos in the litter. The distribution of the mortality before implantation in litters that survive approaches this theoretical distribution sufficiently closely to indicate that the greater part, but not the whole, of the mortality tends to be distributed at random. The remainder of the mortality tends to be concentrated in a small proportion of the litters. Various possible explanations of why there should be a greater probability of loss in some litters than in others can be imagined and have been suggested in the text. Investigation of the distribution of the mortality between the two uterine horns of each pair has shown that one contributory factor is unilateral occlusion of the female tract, either through morphological abnormalities or pathological lesions, but that this does not account for more than a small part of the tendency towards concentration of the loss in some litters.

It has been shown that the proportion of ova lost increases with the number of ova ovulated, that is, with the initial size of litter, in litters that survive. This is surprising, since the data exclude loss after implantation. It is possible to imagine that overcrowding of the uteri with embryos or competition for limited supplies of nutriment might become important factors in determining embryonic survival at later stages of development, but these scarcely could be operative before implantation. Yet it must be concluded that the more fertile females which ovulate the larger numbers of ova provide a less favourable environment either for the fertilization of the ova or for their subsequent early development up to the stage of implantation

The evidence that the loss of ova in litters that survive falls less heavily on animals above the average body weight than on those below it indicates that the mortality is affected by the maternal environment and is not due entirely to genetical or other factors intrinsic in the fertilized ova. Possibly maternal endocrine or nutritional influences are at work. It appears, however, that animals with no milk in the mammary glands actually lose a higher proportion of ova for any given litter size above four than do those with milk. Yet the latter include all animals suckling, and which are therefore sustaining much greater nutritional demands than those which are not. This is difficult to reconcile with the idea that nutritional deficiency, at least of a quantitative kind, is an important factor in prenatal mortality occurring before implantation, since the nutritional demands made on the mother by a litter being suckled must be very much larger than those of unimplanted uterine blastocysts. The idea of endocrine deficiency of the mother as a major factor in prenatal mortality before implantation is more in keeping with the results, since the group which have no milk, and therefore have not recently bred, might be expected to include any with endocrine deficiency.

SUMMARY

1. The relation of the proportion of litters showing loss to the proportion of embryos lost is discussed and is shown to vary according to the way in which the mortality is distributed.

2. The proportion of ova lost before implantation in litters that survive varies according to the number of ova ovulated as the linear regression

$$Y=0{\cdot}01395x+0{\cdot}00895,$$

where $x=$ the initial number of ova in the litter. The loss of ova before implantation in the whole sample of 12,551 ova was 1188 or 9·5%.

3. The proportion of litters suffering loss before implantation in which some embryos survive implantation varies according to the number of ova ovulated as the linear regression

$$Y=0{\cdot}0858x-0{\cdot}1289,$$

where $x=$ the initial number of ova in the litter. The number of litters suffering loss before implantation in which some embryos survive implantation in the whole sample of 2179 litters was 796 or 36·5%.

4. Fewer litters suffered more loss than would be expected if the loss were distributed at random amongst the ova. The sample of litters showing loss is not homogenous, and the probability of loss of ova is greater in some of them than in others.

5. In litters that suffer a loss of two or more ova there is a tendency for the loss to be concentrated in one uterus of each pair. Some or all of this tendency may be accounted for by obstruction of the uterine tract on one side. The number of animals out of a total of 2073 which had lost all the ova on one side, but not on the other, was 92, an excess of 18, or 1·8%, over the expectation on a random basis. The occurrence of loss on one side does not affect the probability of loss on the other side.

6. Not more than 3·64% of litters are lost entirely before implantation and probably not less than 1·00%. The percentage of litters lost entirely before implantation is at a minimum in litters of an initial size of five or six, the most frequent sizes, and at a maximum for litters of the largest and smallest sizes. It is also at a minimum during the height of the breeding season, and is higher at the beginning and end.

7. Animals above the average body weight suffer less loss of ova before implantation in litters that survive than do those below the average body weight. There is no detectable seasonal variation in the loss of ova before implantation in litters that survive.

8. The loss of ova before implantation in litters that survive is heavier in animals with no milk in the mammary glands than in those with milk.

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